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Spatial and temporal control of Rho GTPase functions

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Rho family GTPases control almost every aspect of cell physiology and, since their discovery, a wealth of knowledge has accumulated about their biochemical regulation and function. However, each Rho GTPase distributes between multiple cellular compartments, even within the same cell, where they are controlled by multiple regulators and signal to multiple effectors. Thus, major questions about spatial and temporal aspects of regulation remain unanswered. In particular, what are the nano-scale dynamics for their activation, membrane targeting, diffusion, effector activation and GTPase inactivation? How do these mechanisms differ in the different cellular compartments where Rho GTPases function? Addressing these complex aspects of Rho GTPase biology will significantly advance our understanding of the spatial and temporal control of cellular functions.

Like all regulatory GTPases, Rho family GTPases cycle between an active (GTP-bound) and an inactive (GDP-bound) state under the control of GEFs and GAPs. They additionally cycle between the membrane and the cytosol. Membrane anchoring is conferred by a C-terminal prenyl group and, in some cases, adjacent basic residues.[1] While prenylation is thought to be a permanent modification of Rho GTPases, reversible S-palmitoylation of C-terminal sites has also been described.[2-6] In the resting state, these GTPases are sequestered in the cytosol by RhoGDI, away from activators and effectors. Upon stimulation, they dissociate from RhoGDI and associate with membranes, concomitant with activation by GEFs. It has been proposed that this is a two-step mechanism whereby phosphoinositides,[7-10] proteins such as ERM (ezrin, radixin and moesin), p75 neurotrophin receptor and the tyrosine kinase Etk,[11-13] or phosphorylation of RhoGDI[14] promote dissociation of a GTPase/RhoGDI complex followed by membrane anchoring and activation. However, the rate limiting step of this process in living cells has not been determined.

In the active state, Rho GTPases recruit and activate effector molecules that elicit various biological responses. Finally, the bound GTP is hydrolyzed following association with GAPs, and the GTPase then dissociates from the membrane. Available data suggest that dissociation from the membrane is spontaneous, with RhoGDI inhibiting membrane re-association.[15,16] These 2 regulatory cycles are coupled so that biochemical activation (GTP loading), GDI dissociation and membrane targeting are linked, as they are in the reverse reaction, where the GTPase is inactivated (GTP is hydrolyzed to GDP), dissociates from the membrane and rebinds to RhoGDI. Importantly, both activation and membrane translocation are essential for signaling.[17-19]

While these biochemical steps are well studied, surprisingly little is known about how they proceed in living cells, in particular, how they spatially operate at the molecular level. The earlier observation that Rac and Rho partition into caveolae[20] prompted investigation of the role membrane domains play in Rho GTPase function. These studies suggested that cholesterol-enriched membrane domains (aka lipid rafts) are major sites of membrane binding and signaling by Rac and RhoA.[21,22] Significantly, intracellular trafficking and presence of these domains at the cell surface are adhesion-dependent; detachment of cells from their integrin-mediated contacts results in internalization of a large fraction of the lipid raft components to the recycling endosomes with consequent reduction of ordered domains in the plasma membrane.[22-24] Further, deregulation of this mechanism appears to contribute to anchorage-independence in cancer.[23,25,26] This model was based on (1) the co-localization of Rac with lipid raft markers in cells, (2) the strong dependence on cholesterol for Rac binding to cellular or artificial membranes,[22] and (3) the enhancement of Rac activation and signaling by cysteine palmitoylation,[4] a modification that strongly promotes lipid raft partitioning.[27] Consistent with these ideas, inducing raft localization of the Rac GEF Tiam1 promoted formation of lamellipodia,[28] while targeting Rac to lipid rafts could compensate for the lack of

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endogenous mechanisms of targeting.[29] This model placed lipid rafts at the center of Rac recruitment, activation and signaling.

However, the evidence for this model is based on methods whose spatial resolution is limited, and it is hard to reconcile with both the high solubility of the GTPase in the presence of detergent[30] and the known preference of prenyl groups for disordered, non-raft membranes.[31] Our recent study[32] leads to a more nuanced understanding of the roles of membrane domains in Rac function. First, a FRET-based approach in living cells as well as visualization of Rac binding to the microscopically visible liquid-ordered and disordered phases in artificial bilayers showed that a substantial amount, likely the majority, of membrane-bound Rac exists in disordered regions. This distribution appears to be functionally relevant since forced targeting of Rac to non-raft regions lowered its activity and increased its susceptibility to the Rac GAP β2-chimaerin. These results appear incompatible with prior data demonstrating a requirement for cholesterol in Rac translocation and function. The discrepancy was resolved by the use of supported lipid bilayers *in vitro*, where lipid domains could be resolved by light microscopy. In this system, Rac translocation still required cholesterol but occurred preferentially at the boundaries between ordered and disordered domains. Following translocation, Rac diffused freely and accumulated mainly in the disordered phase. Thus, we propose a model whereby the recruitment of Rac at domain boundaries is followed by its diffusion into both raft and non-raft regions. The active GTPase is likely to encounter distinct effectors in different domains, while in non-raft regions it will encounter GAPs, resulting in de-activation (**Fig. 1**).

Whether translocation also occurs at domain boundaries in living cells remains to be seen. Similarly, whether GAPs other than chimaerin prefer non-raft regions and account for the reduced activity of Rac confined to disordered domains is unknown. Nonetheless, this model leads to some interesting predictions concerning how mechanisms that govern Rac localization to different membrane domains might control its activation state and effector interactions. Local enrichment of palmitoyl transferases is one such mechanism. Rac has a strongly basic sequence at its C-terminus that binds anionic phospholipids, such as PIP_2 and PIP_3, so metabolism of these lipids might also govern the extent of Rac partitioning into rafts.[33] Additionally, membrane domain assembly and disassembly might influence these processes, while proteins that bind at the hypervariable region (a C-terminal sequence that exhibits striking diversity among Rho GTPases) could mask the polybasic region or sterically prevent addition of a palmitoyl group on the neighboring cysteine 178,[34] thereby favoring partitioning into disordered regions. Thus, many possible mechanisms can be envisioned by which diffusion and targeting to different membrane regions could control Rac function.

The presence of Rho GTPases at various cellular compartments such as endomembranes, the nucleus, focal adhesions and the bulk plasma membrane in cell-extracellular matrix and cell-cell contacts adds another layer of complexity.[3,35,36] Whether the same GTPase in these different compartments shows similar behavior with respect to membrane translocation, diffusion, effector interaction, inactivation and membrane dissociation is largely unexplored. Coupling between different compartments is also poorly understood. Diffusion of the GTPase on the membrane surface between compartments has been observed[37] and might influence the GTPase cycle. Membrane trafficking between compartments has also been suggested, for example, activation of Rac by Tiam1 on endosomes resulted in protrusive activity on the plasma membrane and cell migration.[38]

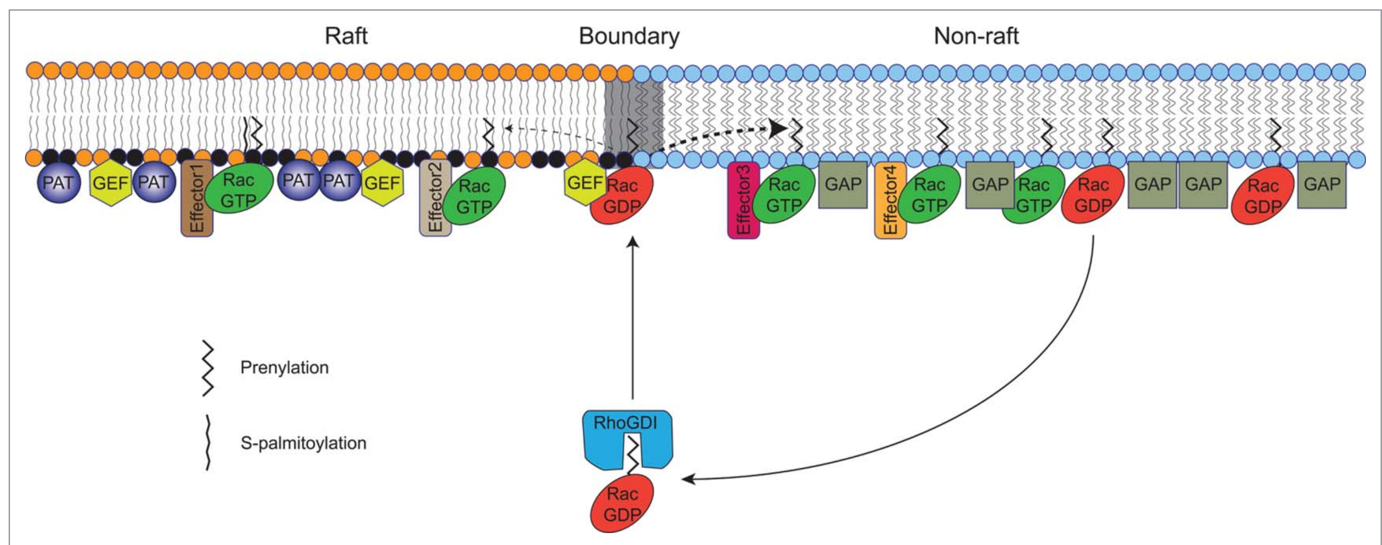

Figure 1. Model of Rac regulation by membrane domains and their boundaries. Rac preferentially translocates at domain boundaries and has a propensity (thick dashed arrow) to diffuse into non-raft regions where GAPs are enriched. S-palmitoylation by palmitoyl transferases (PAT) and interactions with phosphoinositides (black circles) restrict diffusion into raft domains (thin dashed arrow) where the GTPase is activated by GEFs and signals to effectors. Thus, signal termination involves release from raft domains, entry into non-rafts, association with GAPs and membrane dissociation.

Higher complexity still can be appreciated when one considers the multitude of regulators,[39,40] effectors[41] and post-translational modifications described for Rho GTPases.[4,42,43] One view postulates that Rho GTPases at various sub-cellular locations utilize different effectors and thus produce spatially complex signaling outputs. For example, active RhoA is localized not only at the rear of migrating cells but also at the leading edge.[44,45] However, while current models postulate that the RhoA effector ROCK promotes contractility at the tail,[46] active RhoA at the leading edge associates with mDia to stabilize microtubules.[21] Thus, partitioning into ordered vs disordered regions may control the effectors that are activated. Similarly, partitioning into different membrane domains may control GTPase activity by determining interactions with GEFs and GAPs, as we recently reported.[32]

It is becoming apparent that Rho GTPases are controlled by nano-scale reaction and diffusion mechanisms of which we know very little. Understanding these aspects in living cells will require identification of Rho GTPase regulatory elements at various cellular locations and development of new tools to visualize and analyze molecular movements, interactions and reaction intermediates at the single molecule level. Rho GTPases are also highly implicated in disease, including various types of cancer, however, activating mutations are rarely found. Instead, changes in expression levels are often observed.[47] Changes in expression may shift the GTPases between different compartments and effectors, thus, altering the balance between different downstream pathways rather than uniformly increasing or decreasing outputs. In summary, understanding how these changes establish new chemical equilibria and signaling outputs in the Rho GTPase networks that contribute to disease will be important directions for future research.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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