Regulation of endocytic traffic by Rho family GTPases

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Endocytosis is a complicated yet highly efficient process that involves the uptake and processing of cargoes, ranging from small molecules, to activated signalling receptors, to whole microorganisms. Regulation of endocytic pathways is poorly understood. Recent evidence suggests that the Rho GTPase family of signalling proteins is intimately involved in endocytic traffic, providing novel insights into the control mechanisms that govern this process.

The Ras superfamily of small GTPases contains over 80 members. Although the function of many of these remains obscure, they can be grouped into six broad subfamilies – Ras, Ran, Rad, Rab, Arf and Rho – according to a combination of protein homology and function. Proteins in each subfamily tend to have related cellular functions, and the Arf and Rab subfamilies have been shown to have crucial functions in intracellular trafficking, controlling the budding and targeting of vesicles, respectively[1]. The general role of the Rho GTPases seems to be regulation of the actin cytoskeleton[2,3]. However, there is an increasing body of evidence concerning the role of members of this subfamily in a wide range of aspects of endocytic traffic (Fig. 1). Although some of these processes might involve actin rearrangement, others might not. This article aims to present an overview of our, as yet, fragmentary understanding of these events and to propose that the regulation of endocytic traffic might represent a general function of the Rho GTPases.

Internalization: Rac and RhoA

Internalization, the budding-off of membrane vesicles from the plasma membrane, represents the first step of endocytosis. Several different mechanisms of internalization have been described. The most well-studied of these is clathrin-dependent endocytosis, in which receptors are clustered into clathrin-coated pits, which are then pinched off to form coated endocytic vesicles. Activated forms of Rac and RhoA have been shown to block internalization of the transferrin receptor[4], and activated RhoA also blocks internalization of the muscarinic acetylcholine receptor[5]. The potential role for actin in this is unclear. Although actin is not involved directly in the formation of clathrin-coated pits, one mechanistic problem in endocytosis is thought to be the need for internalizing vesicles to negotiate the band of cortical actin that lies directly underneath the plasma membrane. Studies with actin-depolymerizing agents have shown that minor depolymerization of the actin cytoskeleton can stimulate vesicle budding, thereby supporting this idea. Greater depolymerization of actin was found to prevent budding of clathrin-coated vesicles, suggesting that local remodelling of the actin cytoskeleton (i.e. a combination of polymerization and depolymerization) is required[6]. This could represent the site of action of Rac and/or RhoA.

Several clathrin-independent mechanisms of internalization have also been described in mammalian cells. Uptake through these pathways can be stimulated by inhibition of clathrin-dependent internalization[7]; in keeping with this, activated RhoA causes an increase in clathrin-independent endocytosis in *Xenopus* oocytes[8]. Similarly, activated Rac stimulates fluid-phase uptake by pinocytosis[9], and the pinocytic vesicles are coated in the Rac signalling partner, PAK1[10]. Actin rearrangement would seem to be required for the generation of pinocytic vesicles and might also be involved in their intracellular movement. Recent real-time imaging studies with green fluorescent protein (GFP)-tagged actin have demonstrated the existence of pinocytic vesicles within the cell that appear to be propelled by short-lived actin tails[11]. The role of Rho GTPases in the generation of these structures has not yet been investigated.

Another clathrin-independent internalization pathway involves caveolae. The plasma membrane of mammalian cells contains microdomains of concentrated glycosphingolipids and cholesterol, termed lipid rafts. Caveolae are specialized rafts, consisting of pear-shaped invaginations of the plasma membrane, coated with the protein caveolin and associated with actin[12]. Lipid rafts concentrate a large number of receptors and other signalling machinery, and it has recently been demonstrated that the activated forms of Rac and RhoA localize to caveolae[13]. The contribution of caveolae to clathrin-independent endocytosis is unclear[12]; however, as activated RhoA has been shown to increase the number of caveolae[14], one possibility is that RhoA could function to inhibit internalization by sequestering receptors in caveolae and away from clathrin-coated pits.

By far the clearest example of regulation of internalization by Rho family GTPases comes from the specialized case of phagocytosis. Phagocytosis involves the actin-dependent endocytosis of large particles such as bacteria. Caron and Hall have recently defined two distinct mechanisms. In type I phagocytosis, plasma membrane protrusions extend to engulf the particle and drag it into the cell; this is mediated by coordinated actions of Cdc42 and Rac. In type II phagocytosis, particles sink into actin-lined invaginations in the plasma membrane; here, internalization is dependent

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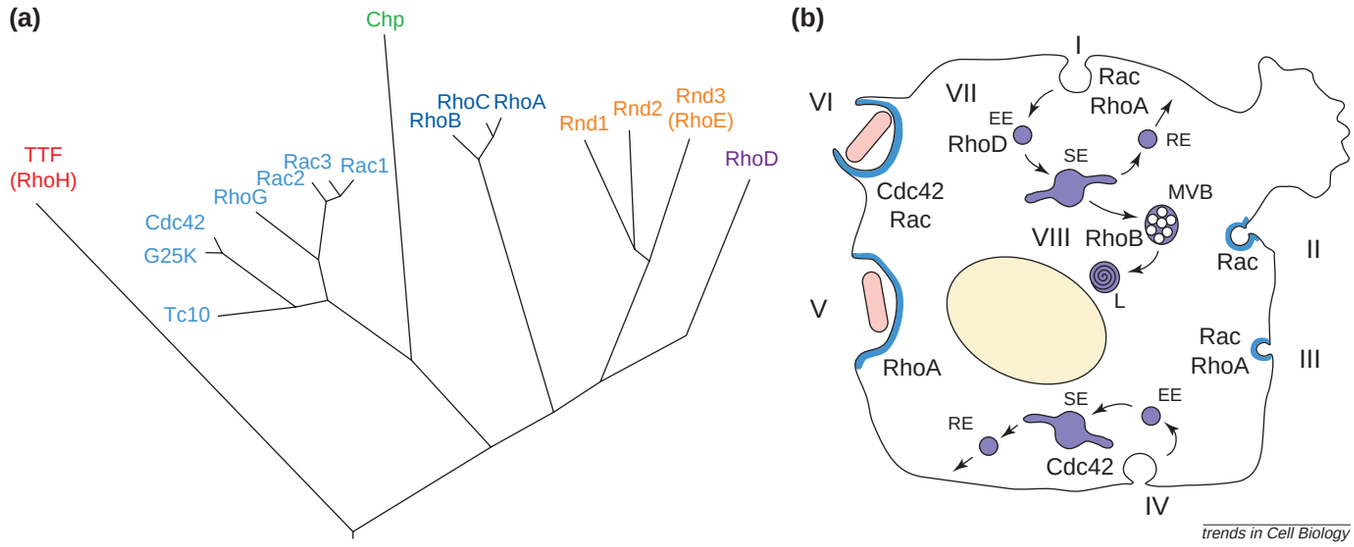

FIGURE 1

Rho family GTPases and endocytic traffic. (a) A dendrogram of the 15 known members of the Rho subfamily of small GTPases (Cdc42 and G25K are splice variants). (b) A cartoon of a hypothetical cell showing all of the known sites of action of Rho GTPases in endocytic traffic (numbered I–VIII); sites of actin polymerization are shown in blue. I, Rac and RhoA inhibit internalization of the transferrin receptor; II, activation of Rac causes production of membrane ruffles or lamellipodia and associated pinocytic vesicles; III, Rac and RhoA are localized to, and cause production of, caveolae; IV, in polarized epithelial cells, Cdc42 regulates endocytic traffic at the basolateral membrane; V and VI, in macrophages, the phagocytosis of opsinized bacteria is mediated by RhoA and Cdc42/Rac in two distinct, actin-dependent processes; VII, RhoD controls early endosomal motility; VIII, RhoB regulates the trafficking of the epidermal growth factor (EGF) receptor to the lysosome. Abbreviations: EE, early endosome; L, lysosome; MVB, multivesicular body; RE, recycling endosome; SE, sorting endosome.

on RhoA[15]. There are striking morphological similarities between these processes and the invasion of mammalian cells by certain pathogenic bacteria. Adhesion of *Salmonella* or *Shigella* bacilli to epithelial cells leads to the production of membrane ruffles at the site of contact; these membrane ruffles are involved in an entry process that is reminiscent of type I phagocytosis[16]. Furthermore, *Salmonella* injects into the host a bacterial protein, SopE, that is an activating GDP–GTP exchange factor (GEF) for host Cdc42. A number of other bacterial toxins that also target Rho family GTPases have been isolated[17], and it would seem that the bacterial subversion of Rho-regulated internalization mechanisms might be widely relevant to invasion.

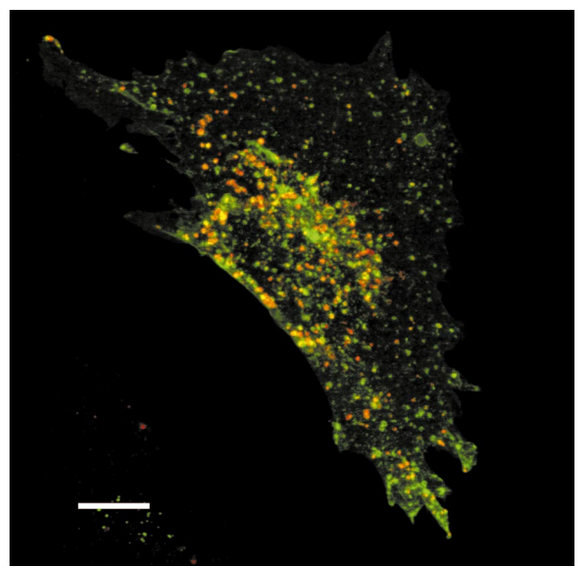

FIGURE 2

Cellular distribution of RhoD and RhoB GTPases. NIH 3T3 cells were cotransfected with cDNAs encoding wild-type RhoB and RhoD. The figure shows a confocal image of cells fixed and stained for RhoB (red) and RhoD (green). Areas of colocalization are seen as yellow. RhoB is absent from the small peripheral RhoD-positive vesicles, which probably represent early endosomes. Bar, 10 μm.

Endosomal Rho GTPases: RhoD and RhoB

On internalization, receptors travel through a number of endocytic compartments, where sorting decisions are made that determine their cellular fate. Some receptors, such as the transferrin receptor, are recycled to the cell surface, whereas others, such as the epidermal growth factor (EGF) receptor, are targeted for degradation in lysosomes. Two members of the Rho GTPase family have been shown to localize to endocytic vesicles, suggesting that they might perform roles in regulating intracellular traffic.

RhoD localizes to vesicles that can mostly be labelled by brief uptake of transferrin, and are therefore likely to correspond to early and/or recycling endosomes[18]. The small GTPase Rab5 has a similar subcellular distribution, and regulates traffic through this compartment by controlling the rate of endosomal fusion[1]. RhoD does not effect the fusion of early endosomes but, instead, regulates the rate of traffic of vesicles along cytoskeletal tracks. Endocytic vesicles are moved along cytoskeletal tracks (usually microtubules) by motor proteins. This is a way of increasing the speed and efficiency of sorting, instead of allowing

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vesicles to find their destination by simple diffusion[19]. Whether the tracks that RhoD-containing endosomes travel on are microtubules or actin filaments is unclear, although endosomes in cells expressing activated RhoD show a striking alignment with actin filaments[18]. It might be expected that reduction of early endosome motility by RhoD would reduce the efficiency of sorting and hence slow the kinetics of receptor traffic. Surprisingly, overexpression of RhoD has only a very small effect on the recycling time of the transferrin receptor[18]. Although this apparent contradiction is confusing, it could be that the actions of RhoD are more relevant to receptors *en route* to other endocytic compartments, such as the lysosome.

The RhoB GTPase, although not closely related to RhoD, also localizes to endocytic vesicles. The precise identity of the RhoB compartment has yet to be defined. RhoB shows some colocalization with internalized transferrin receptor and is absent from lysosomes[20]. Studies of RhoB distribution by electron microscopy show that RhoB is associated predominantly with structures resembling multivesicular bodies[21], a prelysosomal compartment that is thought to be involved in the sorting of internalized receptors for degradation. Interestingly, although RhoB shows some degree of colocalization with RhoD, it is absent from the smaller, peripheral RhoD-positive vesicles (Fig. 2). This observation, and the fact that the EGF receptor does not enter the RhoB-positive compartment until approximately 30 min after internalization[22], suggests that RhoB might be absent from early endosomes.

The precise localization of RhoB would seem key to its function. RhoB is highly homologous to RhoA (83% identical) and shares an overlapping set of binding partners. However, whereas RhoB localizes to endocytic vesicles, RhoA is largely cytoplasmic, translocating to the plasma membrane on activation. This suggests that any specificity of action of RhoB would have to depend on its differential compartmentalization. Evidence for this comes from the finding that RhoB, but not RhoA, regulates endocytic trafficking of the EGF receptor. RhoB retards the progress of the activated receptor through the RhoB-positive compartment on its way to the lysosome for degradation[22]. Mitogenic signals from the EGF receptor (and many other receptors) terminate in the lysosome, and the duration of growth-promoting signals is therefore governed by the time taken for activated receptor to reach this compartment. The regulation of EGF-receptor traffic by RhoB suggests ways in which signalling pathways could be regulated indirectly by controlling intracellular traffic. RhoB expression is rapidly induced by the stimulation of cells with EGF and other growth factors[23], and a marked increase in RhoB protein is seen within 30–60 min of treatment, suggesting that RhoB could mediate both acute and adaptive responses to growth factor stimulation. Some details of RhoB action are known. RhoB regulates traffic through activation of the PRK kinases, members of the protein kinase C superfamily[24]. This suggests that phosphorylation of an endosomal protein is involved; however, the identity of the presumed PRK substrate(s) is unknown. Products of phosphoinositide 3-kinase are also required because activation of PRK involves both RhoB and the phosphatidylinositol lipid-dependent PDK1 kinase (P. Flynn, pers. commun.). This could be important because it suggests a possible basis for at least some of the multiple effects of these signalling lipids on endosomal traffic[25].

Traffic in polarized cells: Cdc42

Polarized epithelial cells present special problems for endocytic traffic. Maintenance of polarity requires that receptors expressed on the apical or basolateral membranes are returned to their appropriate location after internalization. Additionally, epithelial cells possess mechanisms of transcytosis – the endocytic traffic of receptors from one face of the cell to the other. Of all the aspects of endocytosis, the evidence for a role of actin in traffic in polarized cells is the most compelling. Transcytosis is sensitive to the actin-depolymerizing drug cytochalasin D[26,27], and several studies have provided evidence for a role for actin in both apical[28] and basolateral[29] uptake. A role for Rho GTPases in the generation of cell polarity is conserved from yeast to mammalian cells. Underscoring this is the recent finding that Cdc42, which plays a key role in cell polarity in yeast[30], regulates polarized endocytosis at the basolateral membrane of mammalian epithelial cells[31].

Concluding remarks

Of the 15 members of the Rho GTPase subfamily that have been isolated so far, five have already been shown to be involved in the regulation of endocytic traffic. The scope of this article does not encompass other aspects of intracellular traffic, such as secretion, for which there is further evidence of a role for these signalling proteins. The mechanisms by which Rho GTPases exert their effects on endocytosis are still largely obscure, but they seem to represent an interface between signal transduction and membrane traffic. It seems possible that Rho GTPases might be used to mediate acute regulation of endocytic processes.

The extent and nature of the involvement of actin in mammalian endocytosis is also unclear. Studies with actin-depolymerizing drugs have yielded indefinite and sometimes contradictory results. Functional mutants of Rho GTPases are potentially more subtle and discriminating tools to address this question. Hopefully, studies of the mechanisms of action of Rho GTPases in endocytic traffic will clarify this issue, as well as uncovering novel regulatory mechanisms of this fundamental cellular process.

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Pictures in cell biology
Monoastral spindles

Small molecules are able to perturb specific protein functions on a fast time scale and are therefore extremely powerful tools to study dynamic cellular processes such as mitosis. Until recently, this approach was limited by the fact that all known small molecules that specifically affect the mitotic machinery target tubulin, the subunit of the microtubules in the mitotic spindle.

To identify membrane-permeable small molecules that target other mitotic proteins, a combination of two phenotype-based screens, one based on a mitosis-specific posttranslational modification, the other visualizing microtubules and chromatin, and an *in vitro* microtubule-polymerization assay were performed[1]. Out of 16 320 compounds, five small molecules were identified that specifically affect mitosis without targeting tubulin. The phenotype of one compound, named monastrol, was especially intriguing. In BS-C-1 (monkey epithelial kidney) cells treated with this 1,4-dihydropyrimidine-based compound, the bipolar mitotic spindle was replaced by a monoastral microtubule array surrounded by a ring of chromosomes (Fig. 1).

Monastrol specifically and reversibly inhibits *in vitro* the motility of the mitotic kinesin Eg5, a motor protein known to be required for spindle bipolarity. Monastrol will now be used to dissect the function of Eg5 in establishing and maintaining spindle bipolarity. For these studies, it is very useful that this small-molecule inhibitor rapidly induces monoastral spindle formation in cells, allowing an exact temporal control of the induction of and release from the desired phenotype.

For further information, visit the following Web site: http://iccb.med.harvard.edu

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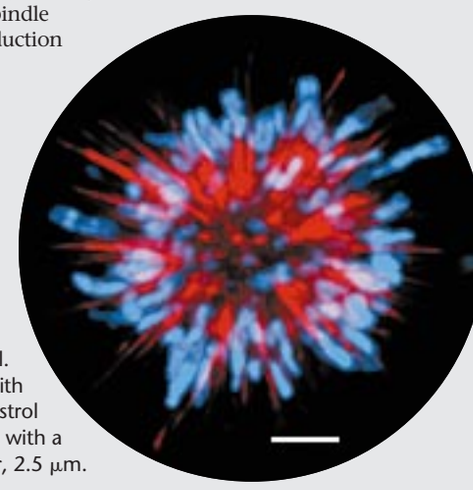

FIGURE 1

Immunofluorescence image of BS-C-1 (monkey epithelial kidney) cells treated for 4 h with 68 μM of the small molecule, monastrol. Microtubules (red) and chromatin (blue) were stained with antibodies to α-tubulin and Hoechst, respectively. Monastrol treatment of mitotic cells replaces the normal bipolar spindle with a rosette-like microtubule array surrounded by chromosomes. Bar, 2.5 μm.

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