REVIEW ARTICLE

Regulation of endocytic traffic by Rho GTPases

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The members of the Rho subfamily of small GTPases are key regulators of the actin cytoskeleton. However, recent studies have provided evidence for multiple additional roles for these signalling proteins in controlling endocytic traffic. Here we review our current understanding of Rho GTPase action within the endocytic pathway and examine the potential points of convergence with the more established, actin-based functions of these signalling proteins.

Key words: actin, endocytosis, migration, phagocytosis, pinocytosis.

INTRODUCTION

Rho GTPases play a pivotal role in the dynamic regulation of the actin cytoskeleton and, through this, control cell morphology, motility and adhesion [1,2]. The human Rho GTPase family comprises at least 23 separate proteins, of which RhoA, Rac1 and Cdc42 are the best studied [3] (Figure 1). Rho GTPases are also present in a wide range of simpler organisms [4], and studies of yeast (*Saccharomyces cerevisiae*), the social amoeba *Dictyostelium*, plants and fruitflies (*Drosophila*) have all shown striking parallels in cellular function [5–8].

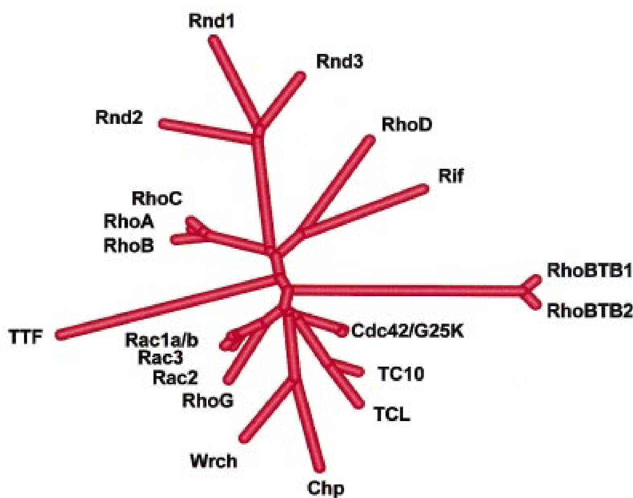

Figure 1 Dendrogram showing the relatedness of the 23 members of the human Rho GTPase family

Sequences were aligned using the ClustalW algorithm with the Gonnet 250 protein weight matrix and displayed in TreeView. Rac1a/b and Cdc42/G25 are the splice variants of the Rac1 and Cdc42 genes respectively. The alternative nomenclature for Rnd3 is RhoE. RhoBTB3 is highly divergent and is excluded from the alignment.

In the last few years, studies in mammalian cells and other organisms have uncovered multiple links between Rho GTPases and membrane traffic. Rho GTPases act locally on individual trafficking events, but they also act globally to control the spatial organization of membrane traffic. One consistent feature is that Rho GTPases are activated in response to extracellular cues, allowing the potential for dynamic regulation of membrane-trafficking processes in response to the extracellular environment. Here we review our current understanding of Rho GTPase function in the endocytic pathway and assess the functional relevance of these regulatory inputs.

CLATHRIN-INDEPENDENT INTERNALIZATION

The most clear-cut interface of Rho GTPases with the endocytic pathway is in clathrin-independent internalization from the cell surface. Recent studies have uncovered a number of clathrin-independent routes into the cell [9] (Figure 2). All of these are driven by actin polymerization and all have obligate requirements for Rho GTPases. The best-studied mechanisms are those of professional phagocytes, which define two separate templates for clathrin-independent entry [10,11].

PHAGOCYTOSIS I

Type I phagocytosis is mediated by Fc receptors which recognize targets opsonized by immunoglobins [12]. Activated Fc receptors recruit Rac and Cdc42 to the phagocytic site [10]. Here, localized Cdc42 activation causes the extension of filopodial projections around the particle, whereas Rac is required for the subsequent particle internalization [13]. RhoA is also recruited, but its necessity for this process is unclear [11]. Castellano et al. [14] have used derivatized latex beads to trigger specific activation of Cdc42 or Rac separately at the plasma membrane. Interestingly, these experiments show that, whilst Cdc42 activation is required for significant filopodial extension [14], Rac activation is sufficient to mediate internalization [15]. The precise requirement for Cdc42, then, is unclear, but it may be that the increased surface contact afforded by filopodia projection increases the efficiency

Abbreviations used: ACK, activated Cdc42-associated tyrosine kinase; Arp2/3 complex, Arp2–Arp3 complex; EGF, epidermal growth factor; GPI, glycosyl-phosphatidylinositol; Hrs, hepatocyte-growth-factor-regulated tyrosine kinase substrate; MTOC, microtubule organizing centre; MVBs, multivesicular late endosomes; PAK, p21-activated kinase; PKC, protein kinase C; ROCK, Rho-associated coiled-coil-containing protein kinase; ROK, RhoA-binding kinase; (N-) WASP, (neuronal) Wiskott–Aldrich syndrome protein.

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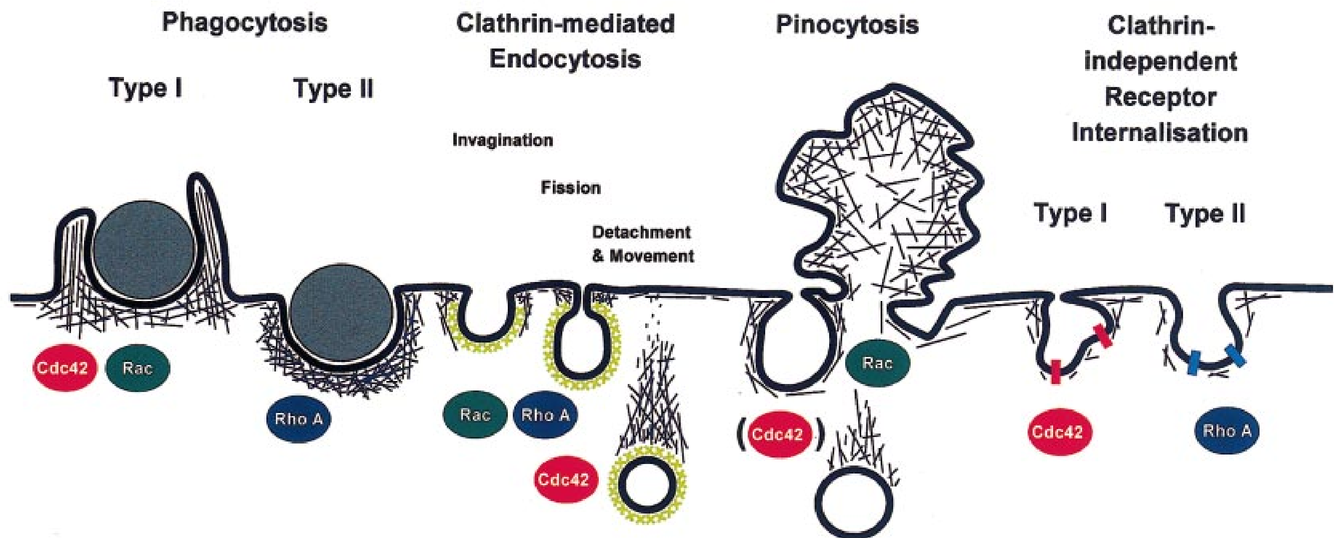

Figure 2 Diagram summarizing the known and proposed sites of action of Rho GTPases in internalization from the plasma membrane

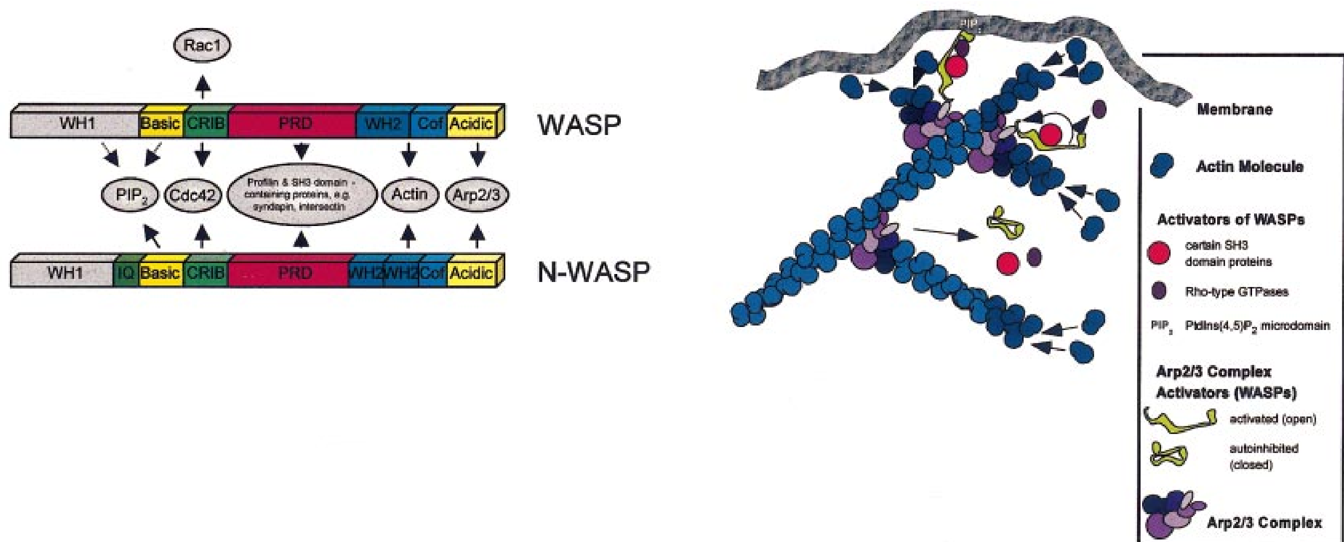

Figure 3 The most prominent family of Arp2/3 actin nucleation complex activators comprising Scar/WAVE proteins and members of WASP family [104]

All these proteins exist in inhibited states. While Scar/WAVE proteins seem to be held in an inactive state by several associating factors, which can be released upon binding of activated Rac1 to these inhibitors, WASPs are autoinhibited. This intramolecular clamp can be released upon binding of activated Cdc42 [105] and other factors, such as PtdIns(4,5)P_2, which synergize in triggering Arp2/3 complex activation *in vitro* [106]. WASPs seem also to be involved in internalization processes. In peripheral-blood cells deficient in WASP FcφR-mediated phagocytosis is impaired [107]. WASP-deficient murine macrophages mirror the phagocytic defect observed these cells and additionally show severe defects in the phagocytic clearance of apoptotic cells [108,109]. The localization of WASP to the phagocytic cup [107] thereby suggests a role in the recruitment and local activation of the Arp2/3 complex actin polymerization machinery. WASPs also seem to be involved in receptor-mediated endocytosis, where the role of actin is not well established. Lymphocytes from mice deficient for the blood-cell-specific WAS protein exhibit both a decrease in actin polymerization and defects in T-cell-receptor endocytosis [59]. Furthermore, WASPs have been shown to be associated with a variety of proteins implicated in clathrin-mediated endocytosis, including Grb2, intersectins and syndapins [110]. Recent studies also provide evidence for an involvement of N-WASP in receptor-mediated endocytosis. Overexpression of N-WASP has dominant-negative effects that are exclusively dependent on the proline-rich domain, whereas other parts of the multidomain protein encompassing, for example, the Cdc42-binding site, do not cause any inhibition of ligand uptake. The N-WASP proline-rich domain-dependent phenotype is completely rescued by co-overexpression of the N-WASP interaction partner syndapin, indicating that syndapins integrate N-WASP functions in endocytosis. Consistently, *in vivo* depletion of endogenous N-WASP suggests an essential role for N-WASP in receptor-mediated endocytosis [111]. F-actin assembly at endocytic sites promoted by N-WASP might first support the pinching-off process, subsequently leading to the detachment and propulsion of the vesicle through the cytoplasm. Elegant live-imaging studies have identified short-lived actin 'comet' tails on intracellular vesicles that power intracellular movement [112] and have recently also revealed that actin associates with endocytic structures upon their detachment from the plasma membrane [60]. N-WASP is present at the interface of endosomes and lysosomes and actin tails in *Xenopus* extracts [112] and is an essential component for actin assembly induced at the surface of vesicles as a consequence of increased PtdIns(4,5)P_2 levels upon type I PtdIns4*P* 5-kinase overexpression [113]. So far, an involvement for Rho GTPases in this process has only been shown with dominant Cdc42 mutants in *Xenopus* extracts [114]. The reconstitution of vesicle movement in the N-WASP-deficient cells by expression of N-WASP mutant proteins reveals an involvement of different domains, including the WH1- and the proline-rich domain, but not the Cdc42-binding domain for this vesicle motility [113]. Since WASP proteins can be activated by different means, e.g. binding of proteins to its proline-rich domain [115], it is still unclear as to whether Rho GTPases are involved in this form of vesicle movement.

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of the subsequent internalization process. The mechanism of actin-dependent internalization involves at least three regulatory steps for Cdc42 and Rac. Both signalling proteins are required for recruitment of the Arp2–Arp3 (Arp2/3) complex to the phagocytic cup, together with its regulator WASP (Wiskott–Aldrich syndrome protein) [16]. Arp2/3 promotes actin nucleation and consequent polymerization at the site of internalization (see Figure 3) and is required for efficient uptake. The type Iα PtdIns4*P* 5-kinase is also recruited to the phagocytic cup, and provides localized PtdIns(4,5)P_2 for regulation of F-actin [17]. This lipid kinase is directly activated by Rac [18]. Finally, the serine/threonine kinase PAK (p21-activated kinase), a downstream effector of both Rac and Cdc42 effector [19], is also targeted to the site [20]. PAK1 increases actin polymerization through inhibition of cofilin [2]. Its contribution to phagocytosis is unclear, although there is some evidence that its signal may require termination to allow closure of the cup [21].

PHAGOCYTOSIS II

In Type II phagocytosis, particles opsonized with the complement molecule iC3b are recognized by phagocytes displaying the CR3 complement receptor (integrin $\alpha_M\beta_2$) [12]. Here, phagocytosis is dependent on RhoA, and not Cdc42 or Rac, and the process is morphologically distinct – particles are seen to sink into the cell with an absence of membrane protrusion [10]. Like Type I phagocytosis, the actin-nucleating Arp2/3 complex is required; however, its recruitment is dependent on RhoA activation and is independent of Rac and Cdc42 [16]. This is an unexpected finding, as currently there are no known pathways linking RhoA to Arp2/3 activation. Recent work has shown that the RhoA effector ROCK/ROK (Rho-associated coiled-coil-containing protein kinase/RhoA-binding kinase) is also required for Type II, but not Type I, phagocytosis [22]. The ROCK/ROK protein kinase regulates actinomyosin contractility [3] and may therefore contribute to the mechanical process of engulfment. Surprisingly, ROCK/ROK is also required for Arp2/3 recruitment. The precise linkage here is unclear, however; Olazabal et al. [22] have suggested that an initial actin assembly promoted by ROCK/ROK may allow subsequent recruitment of Arp2/3 to the phagocytic site.

Ultrastructural examination of the phagocytic cup shows contact with the particle at distinct foci rather than the continuous contact seen in Type I phagocytosis [23]. One of the first functions described for RhoA was the stimulation of focal adhesions, integrin-rich complexes that mediate adhesion to the extracellular matrix [24]. The contact points in Type II phagocytic cups bear a marked similarity to these structures and share many components (e.g. talin, vinculin and paxillin) [23]. This had led to the intriguing hypothesis that Type II phagocytosis may represent an adapted cell-adhesion mechanism [12]. Indeed, CR3 is a functional integrin, capable of mediating adhesion to fibronectin [25]. Focal-adhesion proteins are also recruited to Type I phagocytic cups, but are delocalized within the structure [23]. In keeping with this, Rac and Cdc42 stimulate the formation of focal complexes, a looser organization of adhesive components [26].

‘NON-PROFESSIONAL’ PHAGOCYTES

Phagocytic responses can be induced in cells that are not professional phagocytes simply by expression of the relevant receptors, suggesting that the basic machinery of phagocytosis is present in all cells. The phagocytic clearance of apoptotic cells is of major importance during development, and is a feat that most cells appear capable of carrying out. In the nematode worm *Caenorhabditis elegans* this is a Rac-dependent process [27], and recent work with mammalian cells supports a requirement for Rac and Cdc42 [28,29]. Importantly, these mechanisms are subverted by a number of invasive pathogens, including bacteria [30] and viruses [31,32]. The best studied of these is *Salmonella*, which injects a Cdc42 exchange factor SopE into the host to trigger a phagocytic-like entry [33]. Once inside the host cell, the bacterium manipulates the normal processes of intracellular traffic to form a persistent vesicular structure – the *Salmonella*-containing vacuole – in which to hide out and replicate [34].

MACROPINOCYTOSIS

Phagocytosis handles large cargoes, but comparable mechanisms are being uncovered involving smaller vesicles. The key question is whether these represent scaled-down versions of phagocytic entry. One of the first functions of Rac to be described was the stimulation of macropinocytosis [35]. This actin-dependent event results in the formation of large (0.5–5 μm) vesicles that can deliver cargo to the endocytic pathway or regurgitate it to the extracellular space [36]. The fact that macropinocytic vesicles often form at the base of lamellipodia in actively ruffling cells has lead to the assumption that this is an almost stochastic process of membrane enclosure. However, recent studies have shown that macropinocytosis can be uncoupled from membrane ruffling [37,38], delineating it as a process in its own right and one worthy of serious investigation. Macropinocytosis bears comparison with Type I phagocytosis – it is stimulated by type I PtdIns4*P* 5-kinases and also involves Rac-dependent recruitment of the PAK1 kinase [39]. Importantly, PAK1 activation is both necessary and sufficient for macropinocytosis [40], although determining the mechanistic basis of PAK function is still a key research goal.

Macropinocytosis is the major internalization route for fluid/solutes into the cell. Although the existence of ‘cell drinking’ has been known for some time, we are only beginning to understand its function in multicellular organisms. One important application of this process is seen in dendritic cells. Immature dendritic cells are highly active in both membrane ruffling and macropinocytosis. They use this process to continually sample the extracellular medium, capturing exogenous antigens and storing lysosomally derived peptides in complex with MHC class II molecules. Activation of immature dendritic cells leads to a signalling switch that shuts off macropinocytosis and triggers delivery of antigen–MHC II complexes to the cell surface, where they are presented to T-cells [41]. Macropinocytosis in dendritic cells requires Rac [38,42], and in cells derived from bone marrow it also requires Cdc42 [42]. In an elegant study, Mellman and co-workers [42] have shown that Cdc42 is a key cellular regulator of macropinocytic function in these cells – maturing cells selectively down-regulate Cdc42 activity, and introduction of a constitutively active Cdc42 mutant is sufficient to switch macropinocytosis back on. The situation is not the same for all classes of dendritic cells, however, as macropinocytosis in spleen-derived cells appears to be completely independent of Cdc42 [38].

RECEPTOR INTERNALIZATION WITHOUT CLATHRIN

Two important recent studies have extended our understanding of clathrin-independent endocytosis by identifying two classes of Rho-dependent receptor internalization. Mayor and co-workers [43] have demonstrated a clathrin-independent route for the internalization of glycosyl-phosphatidylinositol (GPI)-anchored receptors. This pathway bears comparison with macropinocytosis in that it is the predominant route for basal fluid-phase uptake in

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the cells studied. The internalization of GPI-coupled receptors, such as the folate receptor, by this route is solely dependent on Cdc42, but independent of Rac [43]. Lamaze et al. [44] have identified a second distinct class of clathrin-independent endocytosis that requires RhoA. So far, the interleukin-2 receptor is the only known cargo, although other candidate receptors exist [9]. It is tempting to speculate that these two types of endocytosis bear a functional relationship to the two classes of phagocytosis. Clearly, our understanding of phagocytic mechanisms provides a range of candidate components for these novel endocytic processes, and there is merit in a comparative analysis. The other key question is whether clathrin-independent receptor internalization is an interesting novelty or a more widespread cellular function. A majority of the candidate clathrin-independent receptor-uptake events occur in free-living cells such as lymphocytes [9]. These are cells with a thick cortical actin cytoskeleton, and Rho GTPase involvement may have a special importance in this situation. Clathrin-independent endocytosis may then represent a means of dealing with specialized circumstances, with clathrin-dependent endocytosis being the more broadly relevant process.

CLATHRIN-DEPENDENT ENDOCYTOSIS

Clathrin-dependent endocytosis is the most familiar entry point to the cell and one of the best-studied membrane-trafficking events. Receptors cluster in clathrin-rich patches at the cell surface that then invaginate to form pits. Constriction of the neck of the pit through the actions of the dynamin GTPase leads to the pinching off of a clathrin-coated vesicle containing the internalized receptor [45]. In contrast with clathrin-independent endocytosis, the actin cytoskeleton is not absolutely required for clathrin-dependent internalization in higher eukaryotes, but instead appears to contribute to the organization and efficiency of the process [46–48]. Similarly, while Rho GTPases do not have an obligate function in clathrin-dependent endocytosis, several studies suggest that these signalling proteins act to regulate the efficiency of internalization.

RhoA AND Rac

Schmid and co-workers [49] were the first to examine the role of Rho GTPases in clathrin-dependent endocytosis and showed that constitutively active mutants of either Rac or RhoA block internalization of the transferrin receptor. However, expression of dominant-negative mutants of these signalling proteins had no effect on receptor uptake in intact cells, suggesting that the observed effects of constitutively active Rac and RhoA may be an indirect consequence of locking these proteins in the active conformation. Symons and co-workers [50] have extended these studies by showing that activated Rac binds directly to the PtdIns(4,5)P_2 phosphatase synaptojanin 2 and recruits it to cell membranes. Localized concentrations of PtdIns(4,5)P_2 act as nucleation sites for endocytosis at the plasma membrane, recruiting clathrin and accessory proteins [51]. After internalization, PtdIns(4,5)P_2 on clathrin-coated vesicles is hydrolysed by synaptojanin, allowing uncoating of the vesicle and the formation of early endosomes [52]. Fusion of synaptojanin to a membrane targeting motif blocks epidermal-growth-factor (EGF)-receptor internalization [50], suggesting that constitutively active mutants of Rac may block endocytosis by recruiting synaptojanin to the plasma membrane and depleting PtdIns(4,5)P_2 at endocytic sites – but what does this say about how Rac acts *in vivo*? Rac is also able to stimulate synthesis of PtdIns(4,5)P_2 through activation of type I PtdIns4P 5-kinases [18,53]. The type I PtdIns4P 5-kinases provide PtdIns(4,5)P_2 for endocytosis [51] and the β-isoform is required for EGF-receptor internalization [54]. The coupling of Rac to both the generation and hydrolysis of PtdIns(4,5)P_2 could therefore allow it to increase overall endocytic rate by facilitating turnover of PtdIns(4,5)P_2 through the cycle. Receptor-mediated activation of Rac is generally rapid, localized and transitory, and these subtle cycles of activity are poorly mimicked by constitutively active mutants. A potentially more informative approach would be to target the exchange factors acting upstream of Rac, and to pick a receptor that (unlike the transferrin receptor) triggers Rac activation. The recent identification of P-Rex1 [PtdIns(3,4,5)P_3-dependent Rac exchanger] as the exchange factor coupling the platelet-derived-growth-factor receptor to Rac activation [55] presents a strong experimental system for testing the relevance of Rac to clathrin-dependent endocytosis.

Cdc42 AND INTERSECTIN

Cdc42 can be linked to clathrin-mediated endocytosis through the multi-domain protein intersectin. Intersectin is a component of clathrin-coated vesicles, and has been termed an 'endocytic scaffold' for its ability to cluster key endocytic components, including Eps15 (epidermal-growth-factor-receptor-pathway substrate 15) and dynamin [56]. The two mammalian intersectins are each produced as two splice variants, with the long forms gaining an exchange-factor domain specific for Cdc42 [57]. Hussain et al. [57] have shown that binding of to the long form of intersectin increases Cdc42 exchange-factor activity. The consequent activation of Cdc42 can then cause neuronal (N)-WASP activation and actin assembly through Arp2/3 [57]. Further, overexpression of an intersectin mutant unable to act on Cdc42 causes severe defects in T-cell-receptor endocytosis that resemble the effects of the actin-sequestering drug latrunculin B [58] or lack of WASP [59] in these cells. This suggests a potential for localized, Cdc42-regulated actin polymerization on clathrin-coated pits. Several authors have proposed that localized actin polymerization could increase the efficiency of endocytosis by providing force for neck closure and/or inward movement of the nascent endocytic vesicle [46,47]. Until recently this has been largely speculative; however, in an elegant study using evanescent-wave microscopy, Merrifield and co-workers [60] have now shown that a burst of actin assembly occurs at the site of dynamin action immediately prior to internalization. Studies with dominant-negative mutants have demonstrated that Cdc42 is not generally required for clathrin-mediated internalization [61]. Such mechanisms may only be necessary under special circumstances – for example, where there is a particularly thick cortical actin cytoskeleton that forms a physical barrier to internalization, and this would be the case with the T-cell study [58]. Another such example is the dense terminal web beneath the apical surface of epithelial cells and, indeed, dominant-negative Cdc42 inhibits uptake of IgA at the apical surface of polarized MDCK (Madin–Darby canine kidney) cells, but not from the basolateral surface [62]. The work of Merrifield and co-workers allows, for the first time, direct visualization of actin assembly at nascent clathrin-coated vesicles, providing a measurable readout to assess the contribution of intersectin/Cdc42 to this process.

Cdc42 AND ACK (ACTIVATED Cdc42-ASSOCIATED TYROSINE KINASE)

The ACK tyrosine kinase is a downstream effector of Cdc42 [63] that also binds directly to clathrin [64,65]. Overexpression of ACK has only modest effects on receptor internalization, which

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are inhibitory or stimulatory depending on expression level [64,65]. However, recent work by Cerione and co-workers [66] suggests that ACK may have more profound effects on events after internalization. ACK expression accelerates the traffic of internalized EGF receptor to lysosomes through phosphorylation of sorting nexin 9 (SNX9)/SH3 and PX domain-containing protein 1 ('SH3PX1'). SNX9 is one of a growing number of factors shown to be involved in deciding the fate of internalized receptors. Interestingly, recent studies have shown that receptors that are marked for degradation are sequestered to a flattened clathrin patch on the face of sorting endosomes [67]. This structure serves as a sorting platform and localizes other sorting factors such as hepatocyte-growth-factor-regulated tyrosine kinase substrate (Hrs) through their clathrin binding motifs [68,69]. It is tempting to speculate that binding of ACK to clathrin at this internal endocytic compartment may be more relevant for the regulation of EGF receptor sorting than binding of ACK to clathrin-coated pits.

ENDOCYTIC TRAFFIC

Internalized receptors travel through a number of distinct endocytic sub-compartments where sorting decisions are made that decide whether the receptor is recycled to the plasma membrane or targeted to lysosomes for degradation. Several Rho GTPases have now been localized to these compartments (Figure 4), suggesting that the actions of Rho GTPases are not confined to the plasma membrane. Whilst we still know relatively little about Rho GTPase signalling from these intracellular compartments, recent studies point to multiple functions for these signalling proteins in endocytic-trafficking pathways.

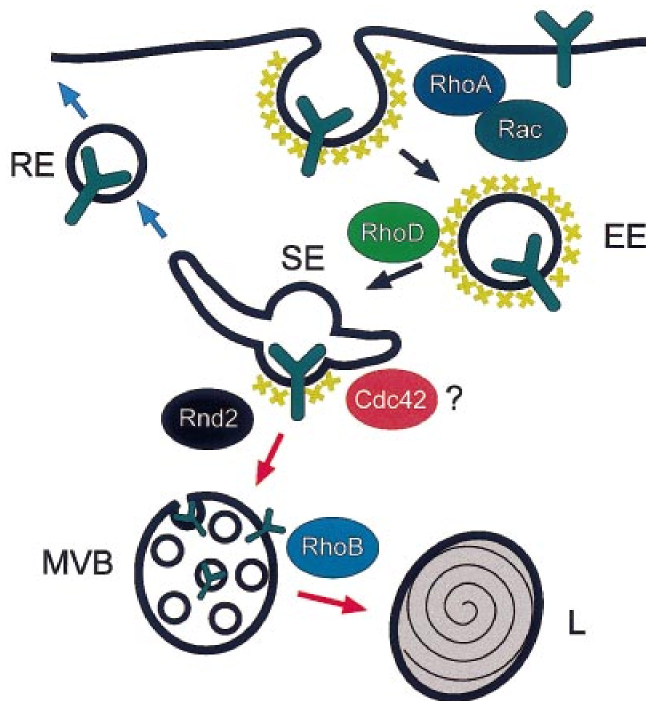

Figure 4 Diagram summarizing the known and proposed sites of action of Rho GTPases in intracellular endocytic traffic

Abbreviations: EE, early endosome; SE, sorting endosome; L, lysosome; RE, recycling endosome.

RhoD AND EARLY ENDOSOMES

The RhoD GTPase is present at the plasma membrane and on early endocytic vesicles [70]. Expression of constitutively active RhoD causes alignment of these vesicles with actin filaments and a loss of early endocytic motility [70,71]. Recent work has shown that RhoD mediates the sequential activation of an isoform of Diaphanous, and the tyrosine kinase c-Src [71a]. The Diaphanous proteins are a subclass of the larger family of formin homology proteins and have previously been shown to be downstream effectors of RhoA [3]. Recent studies have shown that formins can nucleate actin polymerization, providing a second parallel pathway to activation of the Arp2/3 complex [72]. Activation of Diaphanous also causes reorganization of microtubules to form a parallel array with actin microfilaments [73], which would then potentially explain the observed realignment of early endosomes seen with constitutively active RhoD. Dissection of the physiological function of RhoD in early endocytic traffic will clearly require more subtle experimental tools. Given that a proportion of the total cellular pool of Diaphanous protein is localized to endosomes [74], it will be important to determine how targeting Diaphanous function impacts on early endocytic traffic.

RhoB AND LATE ENDOSOMES

Receptors following the degradative pathway are sorted into multivesicular late endosomes (MVBs). The final step in the degradative pathway is fusion between these MVBs and lysosomes, with concomitant transfer of cargo. This transport step appears to be an actin-dependent process [75,76] that has analogies with homotypic vacuole fusion in yeast [77]. In yeast, this latter process is dependent on two Rho GTPases [78,79]: yeast Cdc42 (Cdc42p) and Rho1p. Wickner and colleagues [80] have proposed that the function of Cdc42p is to regulate WASP/Arp2/3-dependent actin remodelling on the surface of the docked vacuoles prior to fusion. The role of Rho1p in docking is less clear, but interesting comparisons can be made to one of its three mammalian counterparts, RhoB. RhoB is highly homologous with RhoA, but, unlike RhoA, is localized both to the plasma membrane and the bounding membrane of MVBs [81,82]. Internalized EGF receptor triggers activation of RhoB as it enters this late-endosomal compartment [83]. Further, expression of activated RhoB impedes traffic of internalized EGF receptor to the lyososome [84], resulting from a block in transfer of the receptor from MVBs to lysosomes (H. Mellor, unpublished work). It is hard to see how blocking receptor traffic in this way serves a physiological purpose, and it would seem that over-expression studies are again highlighting the site of Rho GTPase action, but interfering with its function. The EGF receptor signals to the late-endosomal RhoB pool through Vav2, a guanine exchange factor [83]. This, then, presents a subtler target for dissecting the contribution of RhoB to late-endocytic traffic, and it will be important to determine the effects of down-regulating Vav2 on EGF-receptor sorting.

TC10 AND GLUCOSE UPTAKE

The clearest example of Rho GTPase regulation of endocytic traffic is that of the insulin-stimulated translocation of the GLUT4 (glucose transporter) vesicles. Adipose and striated-muscle cells contain a specialized endocytic compartment that receives a subset of endocytic cargo from recycling endosomes, the most notable being the GLUT4 glucose transporter [85]. In resting cells, GLUT4 can cycle slowly between the plasma

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membrane and GLUT4 vesicles via the endocytic pathway, with the bulk of GLUT4 located within the cell. Insulin stimulation causes rapid fusion of GLUT4 vesicles with the plasma membrane, dramatically increasing glucose uptake. p85/p110 PtdIns 3-kinase is required, but not sufficient to fully activate this process [85]. Chiang et al. [86] have recently shown that the missing component is a pathway involving the Cdc42-related Rho GTPase TC10. The activated insulin receptor triggers a signalling cascade that recruits the exchange factor C3G to plasma-membrane lipid rafts. Interestingly, although C3G is structurally related to Ras exchange factors, it is capable of activating TC10 both *in vitro* and upon overexpression in cells [86].

There are several potential targets for TC10 action. Like Cdc42, TC10 can bind the γ-subunit of coatomer protein I ('COPI') [87]; however, this coat protein is absent from GLUT4 vesicles [88], and this interaction may be more relevant to the function of the perinuclear TC10 pool. Recent studies point instead to regulation of actin remodelling through N-WASP. Insulin stimulation causes the appearance of Arp2/3-dependent actin comet tails on GLUT4 vesicles [89] and TC10 can cause actin-dependent motility of purified endosomes in a reconstituted *in vitro* system [87]. This suggests that TC10 may promote GLUT4 vesicle motility; however, it is important to note that activated TC10 does not clearly stimulate comet-tail formation when expressed in adipocytes [87]. The best-supported function of TC10 is in remodelling of the cortical actin cytoskeleton. TC10 is localized to caveolin-rich lipid rafts in the plasma membrane [90] and recruits N-WASP to these sites upon insulin stimulation [89]. Dominant-negative TC10 blocks the increase in cortical actin polymerization produced by insulin stimulation [89]. Conversely, expression of constitutively active TC10 causes a loss of cortical actin [87]. Taken together, these two seemingly conflicting observations suggest that the function of TC10 is to regulate turnover and remodelling of the cortical actin cytoskeleton at site of GLUT4 vesicle fusion. This should be testable through experiments designed to discriminate between the requirements of TC10 for motility, docking and fusion of GLUT4 vesicles.

MEMBRANE TRAFFIC AND CELL MIGRATION

Spatial reorganization of membrane traffic underlies the generation and maintenance of cell polarity. In budding yeast, Cdc42p plays a key role in polarization and directs secretion to the bud site [5]. In polarized epithelial cells Cdc42 is required both for the targeting of secreted proteins to the basolateral surface and for selective recycling of internalization proteins to this site [61,91]. Polarized membrane traffic is a function of cytoskeletal organization [91], which is, in turn, a target for Rho GTPase regulation. Recent studies on the polarization of migrating cells have shed light on the mechanisms of generation of polarity, and shown that Rho GTPases can acutely regulate the spatial organization of membrane traffic. Migrating cells rapidly polarize membrane traffic towards the direction of movement, targeting both secretion and endocytic recycling to the leading edge [92]. The directed insertion of membrane may contribute to protrusion, but this reorganization also leads to concentration of receptors at the front of the cell, which may reinforce directionality. The first step in polarizing secretion in many cells is realignment of the microtubule organizing centre (MTOC) to face forward, a process that is dependent on Cdc42 [93,94]. Signals from chemoattractants [95] or integrins [96] activate Cdc42 at the leading edge, with consequent recruitment of the atypical protein kinase C (PKC), PKC-ζ, and Par6 (partitioning defective 6). Interestingly, this complex is involved in establishment of polarity in a range of cellular contexts, including key roles in embryogenesis [97]. The target of PKC-ζ phosphorylation in this event is unknown; however, one candidate is the microtubule-based motor dynein, which is also required [96]. This has led to the proposal that reorientation of the MTOC is a result of dynein-dependent 'tug-of-war' mediated through microtubules. MTOC reorientation is accompanied by polarization of microtubules towards the front of the migrating cell. Kaibuchi and co-workers [98] have recently shown that Rac and Cdc42 can mediate microtubule capture at the leading edge through the interaction of the Rac/Cdc42 effector IQGAP with the microtubule tip binding protein CLIP-170. Expression of IQGAP mutants that are unable to bind Rac/Cdc42 leads to the formation of multiple leading edges, demonstrating the importance of this process to directionality. Finally, in polarized migrating cells, microtubules orientated towards the leading edge are selectively stabilized [99]. Microtubule stabilization can occur through RhoA-dependent activation of Diaphanous proteins [73,100] and also through Rac/PAK-mediated phosphorylation of stathmin [101], suggesting ways in which this may be achieved. Taken together, these exciting results imply an overall picture where Rho GTPases co-ordinate cytoskeletal rearrangement with intracellular traffic to generate polarity during cell migration. Clearly, important future work will be to determine if similar mechanisms govern the spatial organization of membrane traffic in non-motile polarized cells such as the asymmetric trafficking pathways of epithelial cells. It is hard to believe that similar mechanisms do not operate.

CONCLUSIONS AND PERSPECTIVES

Recent studies have uncovered a multitude of control points for Rho GTPases in the endocytic pathway. With similar findings in the biosynthetic secretory pathway [102], the resultant picture is one of an intimate connection between these signalling proteins and intracellular traffic. We are used to thinking about membrane traffic in terms of Rab GTPases, proteins that have fundamental roles in defining compartment identity and which are 'hardwired' into trafficking events. Rho GTPase function appears conceptually different, and more concerned with adaptation and plasticity within trafficking pathways. Studies of the workhorse pathways of intracellular traffic have provided a foundation that is now allowing exploration of the more specialized and conditional trafficking events. The evidence to date suggests that it is in these specialized pathways that we will find functions for Rho GTPases. Whilst some of these functions are actin-independent, it is clear that Rho GTPases form a signalling bridge between endocytic traffic and the actin cytoskeleton. The WASPs are an almost ubiquitous presence throughout the pathway and suggest potential input from Cdc42-like small GTPases. As WASPs can be activated through a number of routes, it will be important to determine how many of these WASP/Arp2/3 dependent process actually have an obligate role for Cdc42. Conversely, processes that appear Cdc42-independent may on further investigation turn out to be regulated acutely by Cdc42 under certain specific conditions. Recent studies have shown that Arp2/3 is not the only route to actin nucleation [103] and the interactions of Rho GTPases with formins such as Diaphanous seem likely to emerge as additional bridges between the actin cytoskeleton and membrane traffic. As increasing connections between endocytic traffic and the actin cytoskeleton are revealed [46,47], Rho GTPases would seem to be ideally placed to mediate the signalling interface between these fundamental cell processes.

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