Rho GTPases and their role in organizing the actin cytoskeleton

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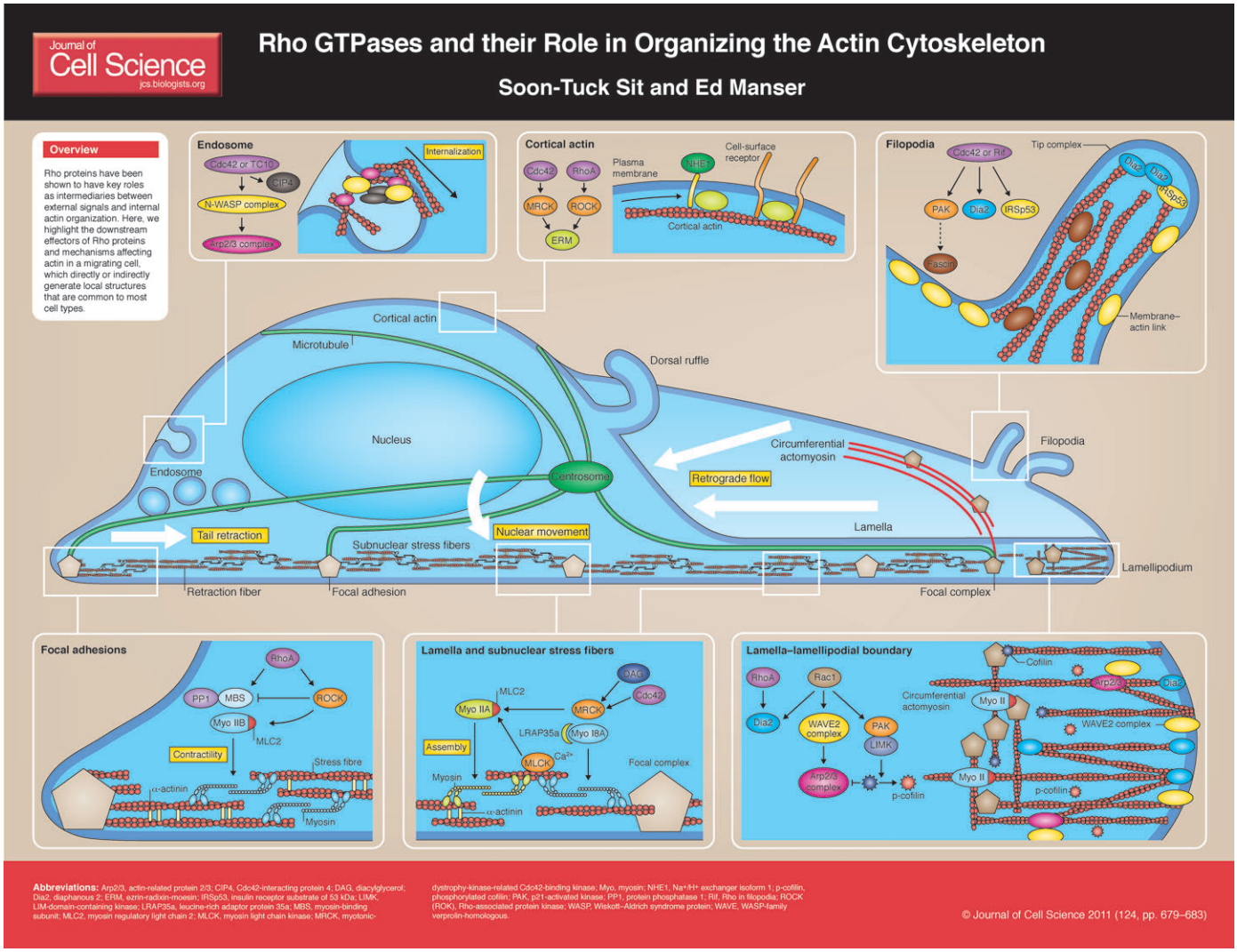
Introduction

Cells receive extracellular stimuli in various ways: in the form of soluble molecules (growth factors, cytokines and hormones) that interact with cell-surface receptors; from adhesive interactions with the extracellular matrix; and from cell–cell adhesions. These stimuli act to generate changes in the actin cytoskeleton at specific sites (see Poster), primarily through Rho proteins. Local guanine-nucleotide-exchange factors (GEFs) or GTPase-activating proteins (GAPs) serve to upregulate or downregulate the active levels of membrane-bound Rho proteins. In humans, ~20 Rho GTPases exist, of which Rho, Rac and Cdc42 remain the best studied (for a review, see Heasman and Ridley, 2008). Once activated, Rho GTPases bind to a variety of effectors, including protein kinases (Zhao and Manser, 2005) and some actin-binding proteins. These directly or indirectly affect the local assembly or disassembly of filamentous (F)-actin. The pathways downstream of Rho that impinge on actin are the subject of this Cell Science at a Glance article. For each cellular structure in a 'typical' adherent cell, we have incorporated information on the specific role of Rho effectors. Some background on these effectors, their regulation and their actin-related roles is provided in the text. This article does not consider the role of Rho proteins in mediating changes in actin dynamics during the cell cycle [for more information on this topic, see Villalonga and Ridley (Villalonga and Ridley, 2006)] or in the specialized podosome or invadopodia structures (for reviews, see Buccione et al., 2004; Linder, 2007; Albiges-Rizo et al., 2009).

The accompanying poster shows a schematic of a migrating cell with a protruding lamellipodium, a thin sheet of cytoplasm that consists mostly of very dynamic F-actin. By using single-molecule speckle analysis of actin filament turnover in lamellipodia, Watanabe and Mitchison (Watanabe and Mitchison, 2002) were able to observe that these F-actin filaments are generated predominantly by polymerization at the lamellipodial tip. There is some controversy as to the arrangement of actin filaments in this



(See poster insert)

region, but the prevailing view was, until recently, that F-actin branching mediated by the actin-related protein 2/3 (Arp2/3) proteins produced a dendritic F-actin network (see Pollard, 2007). This was recently challenged by a study, in four cell types, that concluded that lamellipodial F-actin is almost exclusively unbranched (Urban et al., 2010). This recent study suggests that the majority of 'filament junctions' seen by electron microscopy are in fact overlapping filaments, rather than branched end-to-side junctions. There is agreement that the force that is required for membrane extension is derived from such F-actin assembly, as discussed in the following sections.

The only multiplex live-cell study of RhoA, Cdc42 and Rac1 activation (Machacek et al., 2009) suggests that RhoA acts to initiate protrusive events in the lamellipodial region, whereas sequential Cdc42 and Rac1 activation stabilizes the newly expanded membrane. The region immediately behind the lamellipodium, termed the lamella, is relatively flat and thin (Heath and Holifield, 1991), and actin filaments in this region (some in combination with myosin) undergo retrograde flow towards the cell body. The lamella is distinct from the lamellipodia because of a lower rate of F-actin flux (Ponti et al., 2004) and the presence of actomyosin filaments. Adhesion to the matrix, which is meditated through integrins, occurs both at the lamella–lamellipodial boundary (Kaverina et al., 2002) and at the lamellipodial tip (Alexandrova et al., 2008; Choi et al., 2008). These nascent adhesion complexes tend to mature to become 'focal adhesions' under the influence of sustained myosin II contractility (Vicente-Manzanares et al., 2009). Actomyosin stress fibers link to these adhesions and are essential for their stability. In the lamella region, the stress fibers can be arranged orthogonal to the cell edge (circumferential stress fibers), or they can extend perpendicular to the cell edge towards the nucleus or across the cell center.

Actin polymerization I: promoting F-actin nucleation through formins

Formins were first recognized as Rho targets in a two-hybrid screen with yeast Rho1p, which identified Bni1p (Kohno, 1996). Cells have several strategies for initiating new actin polymerization, of which formins are probably the most commonly used (Nicholson-Dykstra et al., 2005; Rafelski and Theriot, 2004). The mammalian diaphanous proteins (Dia1, 2 and 3; also known as Diap or Diaph proteins) are the best-characterized vertebrate formins, comprising one of the seven formin subfamilies (Chesarone et al., 2010). The defining feature of formins is the FH2 domain and the adjacent variable-length profilin-binding FH1 domain, which cooperatively mediate actin filament assembly. Most formins contain a C-terminal inhibitory autoregulation domain (DAD) and an N-terminal Rho-binding domain (RBD) embedded within the larger inhibitory domain (DID), the latter of which allows auto-inhibition and can be relieved by Rho binding (Rose et al., 2005; Otomo et al., 2005).

The first structure of yeast Bni1p to be determined revealed that the FH2 domain forms a dimeric 'donut' (Xu et al., 2004), which probably fits around the barbed end of the actin filament; a number of subsequent FH2 structures show the same feature. The FH2 domain can remain continually associated (processive) with the barbed end and elongate F-actin in the presence of barbed-end capping proteins, which are abundant in the lamellipodial region. This massive and polarized net assembly of F-actin provides the driving force for membrane extension (Le Clainche and Carlier, 2008; Faix et al., 2009). Although mammalian Dia1 is activated only by the Rho family (i.e. RhoA, B and C), the related Dia2 and Dia3 proteins can also be activated by Rac1 and Cdc42 (Lammers et al., 2008). Evidence for the role of formins in filopodial production has come from a number of sources. *Dictyostelium* Dia2 is enriched at the tips of filopodia, and is needed for formation and maintenance of filopodia (Schirenbeck et al., 2005); the Dia2 protein is similarly enriched at both lamellipodia and filopodia in mammalian cells (Yang et al., 2007). *Ena*- and/or vasodilator-stimulated phosphoprotein (*VASP*)-null neurons are defective in neurite initiation, but can be rescued by restoring filopodia formation through ectopic expression of mammalian Dia2 (Drees and Gertler, 2008). An alternative route for generating new barbed ends involves the assembly of an actin branch on an existing filament – this occurs through the activities of the Wiskott–Aldrich syndrome protein (WASP) and WASP-family verprolin-homologous (WAVE) proteins, as described in the next section.

Actin polymerization II: WASP and WAVE complexes initiate branching

The WASP and WAVE family proteins are fundamental organizers of the actin cytoskeleton in eukaryotes. The Cdc42–Rac-interactive binding (CRIB) domain of WASP was identified by its homology to p21-activated kinase (PAK) (Symons et al., 1996); however, WASP binds Cdc42 but not Rac1. Both WAVE and WASP drive Arp2/3-mediated F-actin branching, and thus rapid actin polymerization, by increasing the number of free barbed ends (Kurisu and Takenawa, 2009). They share a common C-terminal architecture: a proline-rich stretch followed by the 'VCA' region, which triggers actin polymerization through Arp2/3, two actin-like proteins that serve as an actin pseudodimer. The C-terminal domain of ~20 amino acids forms an amphipathic α-helix that activates the Arp2/3 complex (Panchal et al., 2003).

The WAVE2 protein has a selective role in lamellipodial dynamics compared with WAVE1, which has a role in dorsal ruffles (Kurisu and Takenawa, 2009). Both of these WAVE proteins are constitutively incorporated into a heteropentameric complex with Rac1-binding protein PIR121 (also known as Sra1 and CYFIP2), Nck-associated protein 1 (Nap1), Abl-interacting protein (Abi) 1, 2 and 3, and HSPC300 (Gautreau et al., 2004; Innocenti et al., 2004). Careful reconstitution of the WAVE regulatory complex suggests that PIR121 inhibits the WAVE VCA region, so Rac1 activation of the WAVE complex through PIR121 might be intramolecularly similar to Cdc42-mediated WASP activation (Ismail et al., 2009). Maximal activity of the WAVE2 complex requires simultaneous interactions with prenylated membrane-bound Rac1 and acidic phospholipids (Lebensohn and Kirschner, 2009). Phosphorylation of the WAVE complex is also obligatory – for example, WAVE2 requires multiple casein kinase 2 (CK2) phosphorylation events within its VCA domain (Pocha and Cory, 2009).

WASP proteins similarly form a stable one-to-one complex with WASP-interacting protein (WIP) proteins, and require Toca-1 (for transactivator of cytoskeleton assembly 1) (Ho et al., 2004), or its paralogs Cdc42-interacting protein 4 (CIP4) or formin-binding protein 17 (FBP17), for activation. CIP4, similar to the ubiquitous N-WASP, binds to Cdc42-like proteins, but not Rac1 (Aspenström, 1997); the current view is that these proteins primarily promote endocytosis. This idea arose because the yeast WASP homolog Las17p had been identified in a screen for mutants defective in endocytosis (Naqvi et al., 1998). N-WASP probably accelerates actin polymerization near invaginating clathrin-coated pits, providing energy to pinch them off from the plasma membrane. The N-WASP–WIP complex, together with Toca-1 or FBP17, activates actin polymerization on phosphatidylserine-containing membranes (Takano et al., 2008). These roles have been confirmed with the observation that mouse *Cip4*-null cells have delayed and decreased endocytosis (Feng et al., 2010).

Actin depolymerization and severing by cofilin

Once F-actin is formed in the cell, actin filament barbed-end capping regulates the extent of actin

filament elongation. A multitude of such binding proteins influence this process and a number are targets of Rho effector kinases (see Pak et al., 2008). ADF/cofilin proteins are reported to increase subunit dissociation from pointed ends (Carlier et al., 1999); however, other studies counter this notion and suggest that cofilin acts primarily to sever F-actin and block barbed-end elongation (Andrianantoandro and Pollard, 2006). Active cofilin can also dissociate or 'strip off' Arp2/3-derived F-actin branches (Chan et al., 2009). High levels of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] inactivate cofilin in vitro (Yonezawa et al., 1990), but the key regulation occurs by phosphorylation of Ser3 on cofilin by LIM-domain-containing kinases (LIMKs) and testis-specific kinases (TESKs), events downstream of RhoA and Rac1 (Nishita et al., 2005; Huang et al., 2006). This inactivation of cofilin locally promotes F-actin stability and elongation. The Rho-associated protein kinases (ROCKs or ROKs) and PAKs activate LIMK through phosphorylation of its activation loop (Thr508 in LIMK1) (for a review, see Bernard, 2007). The regulation of TESKs is not understood at all. In the lamellipodium, cofilin inactivation depends primarily on activities of Rac1 and its PAK1 and PAK2 targets (Delorme et al., 2007).

MLCK, ROCK, MRCK and PAK regulate the actomyosin network

The Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) phosphorylates myosin regulatory light chain 2 (MLC2) primarily on Ser19 and, to a lesser extent, on Thr18 (Sellers et al., 1981). This affects both activity and assembly of myosin II filaments. In non-muscle cells, MLCK acts on distinct myosin II pools from ROCK (Totsukawa et al., 2004). The long form of MLCK (220 kDa) is dominant in non-muscle contexts, such as in HeLa and PTK cells (Poperechnaya et al., 2000); it contains multiple copies of a DxRxxL motif (not found in the 130 kDa muscle form) that targets MLCK to stress fibers. ROCK family members are direct targets of RhoA (Leung et al., 1995; Fujisawa et al., 1996): this kinase family includes myotonic dystrophy kinase, ROCK, myotonic-dystrophy-kinase-related Cdc42-binding kinase (MRCK) and citron kinase. In addition to binding Cdc42, the MRCK protein kinase C (PKC)-like C1 zinc finger binds, and is activated by, diacylglycerol, thus placing it downstream of phospholipase C (Tan et al., 2001), in common with MLCK. The equivalent ROCK zinc finger might allow regulation by lipids, such as arachidonic acid (Feng et al., 1999), but this has not been fully investigated. Common targets of these zinc fingers are the myosin-binding subunit (MBS or MYPT1) of myosin phosphatase (for a review, see Matsumura and Hartshorne, 2008) and the regulatory MLC2 protein, which controls the activity of myosin IIA and myosin IIB (Vicente-Manzanares et al., 2009). The MBS complex is bound to RhoA, perhaps through the M-RIP adaptor (Kimura et al., 1996; Surks et al., 2003). ROCK also phosphorylates the Na^+/H^+ antiporter NHE-1, which promotes the proton export essential for myosin II action (Vexler et al., 1996; Tominaga et al., 1998).

The phosphorylation and inactivation of the myosin-associated phosphatase PP1δ–MYPT1 complex by both ROCK and MRCK is well established (Conti and Adelstein, 2008) and the notion of localized signaling networks is supported by the direct interaction of RhoA with MYPT1 (for a review, see Ito et al., 2004). Significant direct phosphorylation of the regulatory Ser19 on MLC2 by ROCK and MRCK is also now accepted (Vicente-Manzanares et al., 2009). To date, no phosphorylation-specific antibodies have been reported for active forms of ROCK or MRCK, restricting information on the spatial activation of these kinases in cells. However, the effects of ROCK can be inferred from the use of the somewhat selective kinase inhibitor Y-27632 (Uehata et al., 1997).

In HeLa and U2OS cells, lamellar circumferential actomyosin filaments are enriched for myosin IIA and myosin 18A, and their assembly and movement are controlled by MRCK, not ROCK (Tan et al., 2008). Myosin 18A is an unusual myosin with an actin-binding site N-terminal to the head domain (Isogawa et al., 2005) and a PDZ domain that binds the adaptor complex LRAP35a–MRCK. Knockdown of myosin18A in fact prevents the assembly of myosin IIA in the lamella region (Tan et al., 2008). MRCKα and β are ubiquitous Cdc42 effectors; however, a common alternatively spliced MRCKα isoform with two tandem CRIB domains preferentially binds Rac1 (Tan et al., 2003). Their link to Cdc42 and cell polarity is discussed below.

Cell asymmetry and centrosomal polarity in cell migration

Myosin II is important for the polarity of migrating cells; contractility initiates the breakage of cell symmetry by forming the prospective rear, or tail, of the cell (for a review, see Vicente-Manzanares et al., 2009) and protrusion at the opposite 'leading edge' (Vicente-Manzanares et al., 2007). In migrating cells, the activity of RhoA and ROCK is needed for proper regulation of tail retraction (Pertz et al., 2006). Centrosomal polarity, by contrast, requires the activity of the Cdc42–Par6–aPKC–Par3 complex (Suzuki and Ohno, 2006). Cell polarity is most frequently assessed in cell scratch assays performed on confluent cell monolayers. The wound-edge cells orient their centrosomes towards the wound, a process that is easy to monitor. An existing model of this process invokes that the new extracellular matrix adhesions recruit PAR polarity proteins to the leading edge (Schlessinger et al., 2007). However, by using cells grown on micropatterns, it has been shown that it is cell–cell contacts, rather than new integrin engagement, that allows polarization (Desai et al., 2009). Wounding initially induces displacement of the nucleus towards remaining cell–cell contacts and away from the free edge. Although the centrosome is tethered by microtubules attached to the plasma membrane, this nuclear displacement and subsequent movement requires contractile myosin II driven by the Cdc42 effector MRCK (Gomes et al., 2005), which probably operates through the MRCK-enriched subnuclear stress fibers.

It is notable that fibroblast migration through a three-dimensional extracellular matrix requires ROCK, whereas carcinoma cells rely primarily on MRCK (Gaggioli et al., 2007); in other contexts, PAKs are implicated in cancer cell migration (Molli et al., 2009). The best evidence for overlapping roles of these three kinases comes from experiments in *Caenorhabditis elegans* (which only has single copies of ROCK, MRCK and PAK), where the combined loss of either ROCK and PAK, or ROCK and MRCK, completely prevents the embryonic elongation due to myosin II. This is persuasive genetic evidence for interplay between these three kinases in this conserved pathway (Gally et al., 2009). In addition to controlling actomyosin contractility, ROCK and MRCK promote tethering of actin to cellular membranes, as discussed below.

Tethering F-actin to different membranes through ERM, CIP4 and IRSp53

The bulk of the plasma membrane is tethered to an underlying mesh of cortical F-actin. ROCK and MRCK phosphorylate ezrin-radixin-moesin (ERM) family proteins to activate their function as linkers between F-actin and multiple transmembrane proteins, which stabilizes the cortical actin network (Niggli and Rossy, 2008). In their inactive conformation, the N-terminal FERM domain binds to the C-terminal actin-binding domain. Both PtdIns(4,5)P_2 binding to the FERM domain and ROCK phosphorylation of a crucial threonine residue stabilizes the unfolded conformation, allowing the binding of the FERM domain to transmembrane receptors. Phosphorylation of ERM proteins at filopodia induced by the Cdc42–MRCK complex has

been reported (Nakamura et al., 2000). PtdIns(4,5)P_2 is a highly charged lipid that activates or inactivates many actin-binding proteins (Janmey and Lindberg, 2004). Both Rac1 and RhoA bind to phosphatidylinositol-4-phosphate 5-kinase (PIP5K); this markedly stimulates PtdIns(4,5)P_2 synthesis by all three PIP5K isoforms (Weernink et al., 2004). Unfortunately, the underlying regulation of the Rho–PIP5K pathway remains unclear.

Membrane-binding BAR domains can serve as sensors or promote membrane curvature (Peter et al., 2004). The CIP4 family proteins are components of the N-WASP complex (Tian et al., 2000; Tsujita et al., 2006) and contain an F-BAR domain that probably stabilizes invaginations of the plasma membrane, such as those generated during endocytosis. The Cdc42-related TC10 protein recruits CIP4 during insulin-stimulated Glut4 translocation in adipocytes (Chang et al., 2002). Remarkably, in vitro data indicate that F-BAR proteins and the N-WASP–WIP complex have the ability to 'sense' membrane curvature and couple this information to local actin polymerization (Takano et al., 2008). The F-BAR domains of FBP17 and CIP4 also form filaments through end-to-end interactions that might serve to drive membrane tubulation (Shimada et al., 2007).

Filopodia are abundant rod-like cellular protrusions that are formed by the assembly of actin filaments bundled by fascin and assembled from a 'tip complex'. Filopodia can be induced by Cdc42 or Rif, and require an effector protein called insulin receptor substrate protein of 53 kDa (IRSp53) (Faix and Rottner, 2006). IRSp53 has an inverse (I)-BAR domain that binds to and stabilizes 'bent' cellular membranes (in an opposing direction to CIP4) on protrusions such as lamellipodia and filopodia (Scita et al., 2008). Through its SH3 domain, IRSp53 can form a complex with WAVE2 in vivo (Suetsugu et al., 2006), or with the F-actin capping protein Eps8 (Disanza et al., 2006). The localization of IRSp53 to either lamellipodia or filopodia requires an SH3 interaction, allowing it to promote membrane bending: this is negatively regulated by phosphorylation and binding of 14-3-3 proteins (Robens et al., 2010), which regulate filopodial lifetime.

Conclusions

It is probable that the bulk of Rho effectors have now been uncovered, but much remains to be discovered about how these proteins organize actin. The main challenges are to understand the actions of these proteins in their proper cellular context and to determine the in vivo substrates for the kinase effectors of Rho proteins [including PKN (protein kinase N) and MLK (mixed-lineage kinase), which we have not discussed here]. The hope is that the actin cytoskeleton is regulated by a modular set of macromolecular machines that operate in a similar manner in multiple cell types. Thus, the results obtained so far from a variety of cells grown in two-dimensional cultures would be pertinent to whole tissues and organisms.

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