

Minireview

Rho effectors and reorganization of actin cytoskeleton

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Abstract The small GTPase Rho regulates several actomyosin-based cellular processes such as cell adhesion, cytokinesis and contraction. The biochemical mechanisms of these actions remain unknown. Recently, several GTP-Rho binding proteins were isolated. Among them, p140mDia and p160ROCK appear to work as Rho effectors mediating its action on the cytoskeleton. p140mDia induces actin polymerization by recruiting an actin binding protein, profilin, to the site of Rho action. p160ROCK induces focal adhesions and stress fibers by activating integrin and clustering them by the use of myosin-based contractility.

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Key words: Rho GTPase; Cell adhesion; Actin polymerization; Focal adhesion; Stress fiber; Contractility

1. Introduction

Rho, a Ras homolog of small GTPase, is present from yeast to mammals. It shuttles between the active GTP-bound form and the inactive GDP-bound form and works as a switch in the cell. Its actions have been examined by inactivating endogenous Rho by botulinum C3 exoenzyme which specifically ADP-ribosylates this protein and by microinjecting or expressing dominant active forms of this protein in cultured cells. These studies revealed that Rho is involved in many actin-based cellular processes as well as in nuclear signalling (review [1] and references therein). However, the biochemical mechanisms of these actions remain unknown. Recently, several molecules showing selective binding to the GTP-bound form of Rho have been isolated and proposed as putative downstream Rho effectors. More recent analyses of functions of some of these proteins have provided clues to construct a model of a Rho action on actin cytoskeleton. In this review, we first summarize features of Rho actions on actin cytoskeleton and then discuss recent studies on Rho effectors. A model of the biochemical mechanism of the Rho actions on actin cytoskeleton is presented based on these discussions. Actions of Rho on cell growth, transformation and nuclear signalling are not reviewed here.

2. Rho actions and actin cytoskeleton

Three categories of Rho-mediated actin-based processes have been described. They are cell adhesion to the extracellular matrix, cytokinesis and smooth muscle contraction. Involvement of Rho in the first process was characterized in

detail in fibroblasts, lymphocytes and blood platelets [2–4]. All of these cells are stimulated by external stimuli to activate their integrins to bind their ligands, either extracellular matrix proteins such as fibronectin and fibrinogen or counter-receptors on other types of cells such as ICAM. This process is seen as fibroblast adhesion to substratum, platelet aggregation or lymphocyte adhesion to endothelium. On binding its ligand, the integrin forms a multi-protein complex with various cytoskeletal proteins at its cytoplasmic side and this complex is ligated to actin filaments. These complexes are clustered to form large aggregates in the cell surface and actin filaments are bundled. These structures are seen as focal adhesions and stress fibers, respectively, in cells cultured on dish. Cell adhesion and formation of these structures are inhibited by prior treatment with C3 exoenzyme and induced by injecting Val¹⁴-Rho, a dominant active mutant [2]. Similarly, platelet aggregation and lymphocyte adhesion are also inhibited by inactivation of Rho [3,4]. Because inactivation of Rho does not affect the transmembrane signalling of these stimuli, Rho works downstream of the second messenger to induce the changes in integrins, cytoskeletal proteins and actin fibers. Thus, there appear to be two kinds of processes governed by Rho in this actin cytoskeleton rearrangement. One is integrin activation, and the other is clustering of integrin complexes and actin filaments. Recent studies indicate that the latter process is mediated by myosin-based contractility [5,6]. A question frequently asked is whether integrin activation and clustering are independent events. It is often argued that clustering occurs first and is enough for activation. While this point has not been fully settled, previous experiments using inhibitors such as cytochalasins and staurosporine [4,7] indicate that Rho-mediated cell adhesion can occur without clustering. Cell adhesion to substratum and formation of focal adhesions and stress fibers occur on the ventral surface of cultured cells, but Rho also induces actin cytoskeleton at other sites of the cell. That is typically seen in cytokinesis. Involvement of Rho in cytokinesis was first suggested by an increase of multinucleate cells in cultured cells treated with C3 exoenzyme [8] and subsequently analyzed in detail in cell division of fertilized oocytes from sea urchin [9] and *Xenopus laevis* [10]. These studies revealed that Rho works as a switch between the nuclear and cytoplasmic divisions and is required to induce and maintain the contractile ring, a structure performing cytokinesis. The contractile ring is an actin-based structure formed in the cleavage furrow. Its formation requires de novo actin polymerization and cytoplasmic division is carried out by myosin-based contractility generated in this structure. Rho therefore appears to carry out three kinds of actions, i.e. actin polymerization, actomyosin bundle formation and generation of contractility, at the cleavage furrow. Among these actions, a direct role of Rho in the generation of

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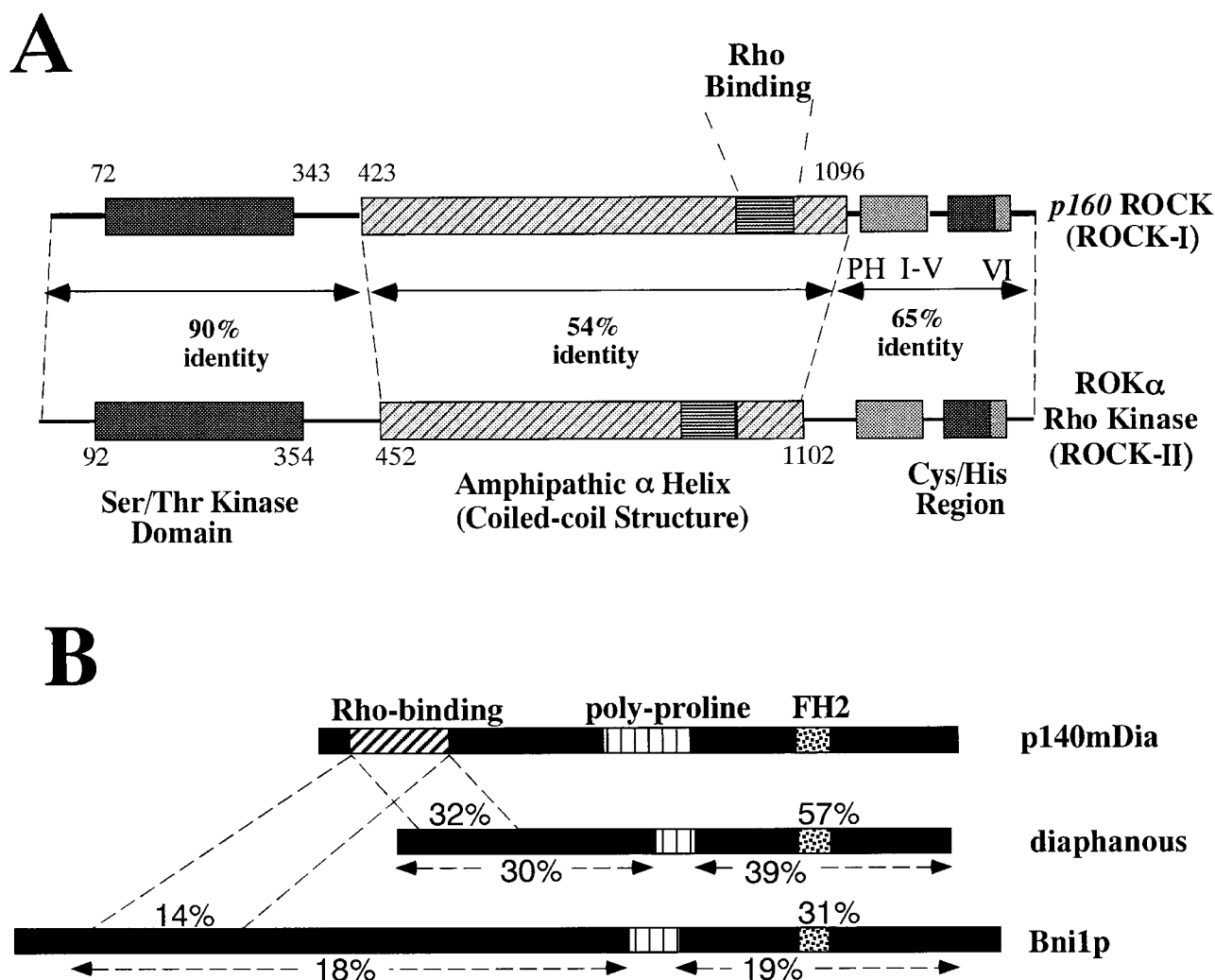


Fig. 1. Structures of Rho effectors. A: A p160ROCK family of kinases. Domain structures of p160ROCK and its isozyme (ROCK-II/ROK α /Rho-kinase) are shown. Amino acid sequence identities of each domain between the two molecules are indicated. B: A family of diaphanous-related molecules. Domain structures of p140mDia, *Drosophila* diaphanous and Bni1p from *Saccharomyces cerevisiae* are shown. Amino acid sequences are compared in regions excluding the poly-proline stretches and in the putative Rho-binding regions and the so-called FH-2 regions.

contractility has been shown clearly by studies on smooth muscle contraction. Smooth muscle contraction is triggered by agonist stimulation, which raises the free calcium ion concentration in the cell, and thereby mobilizes a kinase cascade to phosphorylation of myosin light chain. It was known previously that smooth muscle contraction is determined not only by the level of free calcium ion in the cell but also by a GTP-dependent mechanism that enhances contraction at a fixed concentration of calcium [11]. The latter mechanism is known as 'GTP-induced increase in calcium sensitivity'. Using permeabilized preparations of mesenteric artery, Takai and collaborators identified that Rho is responsible for this induction [12]. Subsequent studies further clarified that Rho suppresses MLC phosphatase activity and thus enhances the level of phosphorylated myosin to increase contractility [13], and that the phosphorylation of the p130 regulatory subunit of MLC phosphatase is involved in the regulation of phosphatase activity [14]. As discussed, Rho-mediated enhancement of contractile responses is seen also in non-muscle cells. Cell contraction is frequently seen when the activated form of

Rho is expressed or injected in cultured cells [15]. Rho-dependent contraction was most clearly found in cultured neuronal cells extending long neurites. These cells retract neurites upon exposure to thrombin or LPA. This is a Rho-dependent process and is apparently caused by tension produced by the actomyosin system [16].

The above discussions reveal three major components of Rho actions: actin polymerization, contraction and site selection. The two former are common in many Rho-mediated processes, but the latter is different depending on the cell type and on the phase of cell cycle. A question is how Rho exerts these components of action and if there are separate effectors mediating each action.

3. Isolation and function of Rho effectors

Initial studies on the mechanism of Rho actions were directed to characterization of downstream pathways of Rho either pharmacologically using various inhibitors or by identifying biochemical changes induced by Rho activation and/or

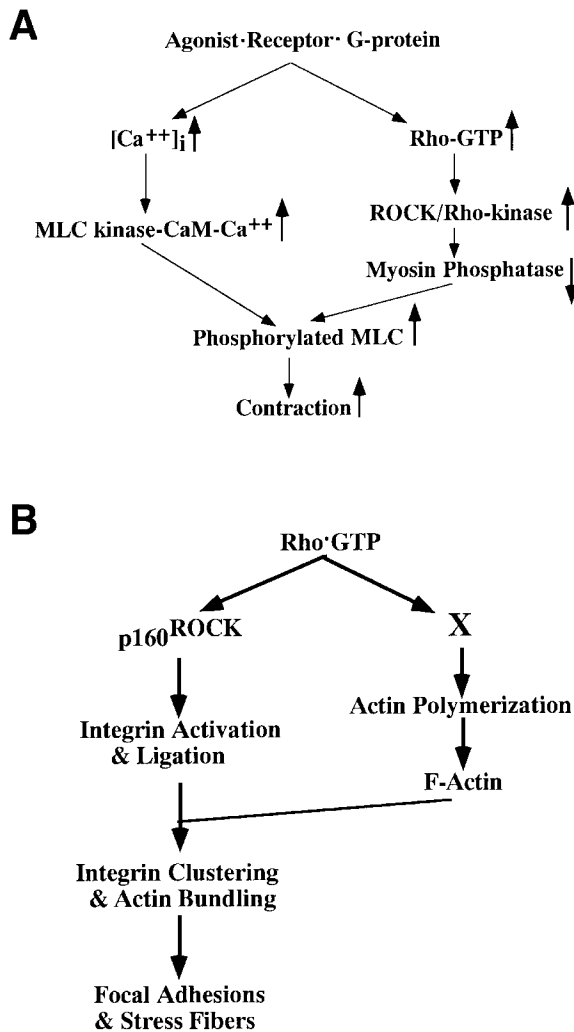


Fig. 2. Actions of ROCK/Rho-kinase in stimulation of myosin-based contractility (A) and in Rho-induced formation of focal adhesions and stress fibers (B). In B, ROCK is proposed to act in two steps, i.e. in activation of integrin and in integrin clustering and actin bundling. In addition to ROCK, an unidentified target (X) mediates Rho action on actin polymerization.

inactivation in intact cells and cell homogenates. These studies revealed that several protein kinases [17–19] as well as lipid kinases such as PI 3-kinase [17,20] and PI 5-kinase [21] are mobilized downstream of Rho. While these findings were implicative, it was essential to identify direct target molecules of Rho to fully define the biochemical mechanism of Rho-induced actin cytoskeleton reorganization. Recently, several proteins showing selective binding to GTP-Rho have been isolated by the use of various methods, such as ligand overlay assay, affinity chromatography and yeast two hybrid screening, and proposed as potential Rho targets [22–31]. Two of these proteins possibly mediating Rho action on the actin cytoskeleton, p160ROCK and p140mDia, are reviewed below.

3.1. Structure and function of p160ROCK, a Rho-associated coiled-coil forming kinase, and its isozyme

p160ROCK [27] is a 160 kDa protein serine/threonine kinase, containing a kinase domain in its N-terminus, a long amphipathic α -helix capable of forming a coiled-coil structure in the middle and the pleckstrin homology domain split by a

cysteine-rich zinc finger in the C-terminus (Fig. 1A). This kinase shows significant homology in its N-terminus to myotonic dystrophy kinase, the product of a causative gene of myotonic dystrophy. A homolog of p160ROCK (Fig. 1A) was isolated and called either ROCK-II [29], ROK α [26] or Rho-kinase [28]. The Rho-binding domain in these proteins is localized between the coiled-coil forming region and the C-terminal PH region [26,32]. Because the former region is presumed to make a coiled-coil complex with other protein(s) and the latter to participate in membrane localization, binding of GTP-Rho to the above site may facilitate these interactions and help their translocation to the target site of the cell. Indeed, ROK α was translocated to the membrane in COS cells co-expressing Val¹⁴-Rho [26]. In thrombin-activated platelets, a part of endogenous p160ROCK is translocated to the cytoskeleton in an integrin-dependent manner (Fujita et al., unpublished observation). Because a homolog of this kinase, MDPK, is localized in tissues at structures analogous to focal adhesions in cultured cells such as dense plaques of smooth muscle, involvement of this group of kinases in Rho-induced cell adhesion was postulated [27]. Clues to their functions have come from two lines of experiments. Kimura et al. [33] have found that Rho-kinase (ROCK-II) is associated with a p130 binding subunit of myosin phosphatase and can phosphorylate this subunit thereby suppressing a phosphatase activity. It has thus become clear that this family of kinases are responsible for Rho-mediated enhancement of myosin-based contractility (Fig. 2A). The second clue has come from expression of these kinases in cultured cells. Leung et al. [34] microinjected cDNA of ROK α into HeLa cells and examined cell morphology. They found that expression of full-length ROK α and its C-terminal truncation mutants induced both focal adhesion-like structures and stress fiber-like actin bundles in the cells, and this morphological phenotype required the kinase activity. Ishizaki et al. [35] reported similar morphological changes in HeLa cells transfected with wild type and various mutants of p160ROCK. Focal adhesion- and stress fiber-like structures became condensed with C-terminal truncated mutants and the cells became contracted, suggesting that myosin-based contractility is exerted and gets stronger with these truncations. They further used a kinase-negative, Rho-binding-defective mutant as a potential dominant negative form and examined its role in Rho-mediated response. This double mutant blocked Rho-induced formation of focal adhesions and stress fibers, indicating that p160ROCK indeed works downstream of Rho. Interestingly, Rho still induced enhancement of F-actin formation, suggesting that Rho-induced actin polymerization is mediated by a different effector (Fig. 2B). This dominant negative mutant, on the other hand, did not suppress Rac-induced membrane ruffling, excluding a recent conjecture by two groups [36,37] that p160ROCK and ROK α may work downstream of Rac.

3.2. Structure and function of p140mDia, a mammalian homolog of *Drosophila diaphanous*

The above study using a dominant negative form of p160ROCK indicates the presence of a different effector mediating Rho-induced actin polymerization. Recently, candidate molecules of this effector have been cloned by two laboratories. They are Bn1p of *Saccharomyces cerevisiae* [30] and p140mDia [31]. As shown in Fig. 1B, the two show significant homology to each other. They have the Rho-binding domain

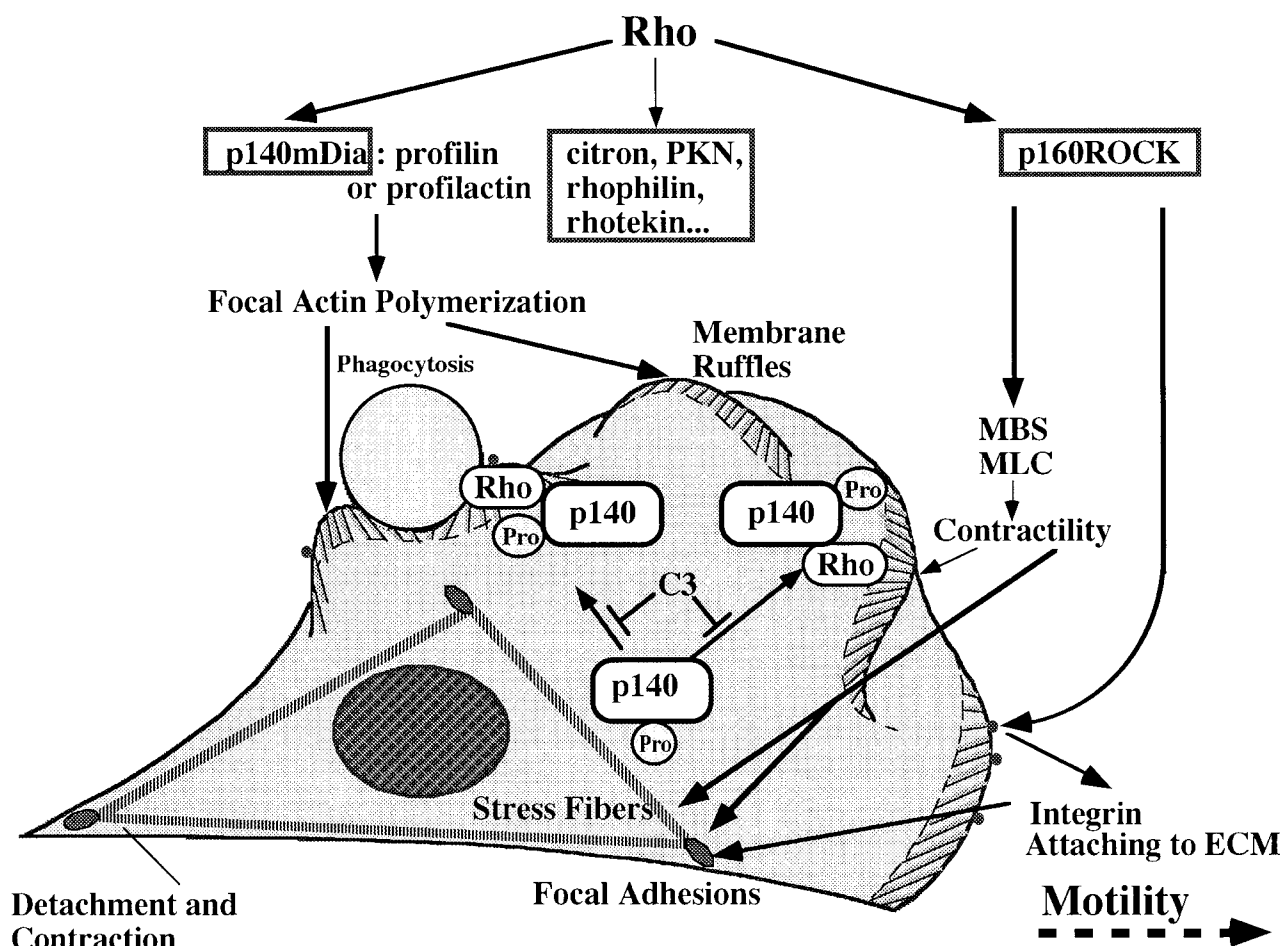


Fig. 3. A hypothetical model of the mechanism of Rho action on actin reorganization. p160ROCK and p140mDia appear to play major roles in this action. Other effectors (citron, PKN, raphilin and rhotekin) may mediate other actions of Rho.

in the N-terminus, a repeated poly-proline stretch in the middle, and the so-called formin homology-2 (FH-2) region in the C-terminus. They also show significant homology to the *Drosophila* protein, diaphanous [38], in the entire sequence, from which the name of mDia (a mammalian homolog of diaphanous) is derived. Interestingly, previous genetic studies indicated that Bni1p and diaphanous are involved in yeast budding and cytokinesis, respectively [38,39]. Watanabe et al. [31] have found that p140mDia binds both to GTP-Rho and to an actin binding protein, profilin, in vitro, and that the three proteins are co-localized in vivo in membrane lamellae of spreading cells. Rho, profilin and p140mDia are also recruited together to phagocytic cups by fibronectin-coated beads in a Rho-dependent manner. Watanabe et al. further found that transient expression of p140mDia induced homogeneous actin filament formation in COS cells. They discussed this action that p140mDia accumulates profilin to the membrane, which in turn stimulates actin polymerization [31]. Profilin, known as an actin monomer binding protein, was thought to function to sequester unpolymerized actin. However, recent studies indicate that it promotes actin polymerization in vitro and in vivo [40,41]. Interestingly, a part of immunoreactive p140 was also observed in the cleavage furrow of dividing cells, indicating that this protein also works in the contractile ring formation.

4. A model of biochemical mechanism of Rho leading to organization of actin cytoskeleton

Based on the above discussions, we envision the downstream signalling of Rho in the reorganization of actin cytoskeleton as shown in Fig. 3. In this model, p160ROCK activated by GTP-Rho first acts on integrins to cause integrin binding to extracellular matrix protein and initial membrane spreading. p140mDia then comes into the extending membranes and recruits profilin, which induces local actin polymerization. Ligand-bound integrins then form a complex with cytoskeletal proteins, which binds actin filaments. Myosin associated with these actin filaments is then stimulated by p160ROCK to contract to cluster ligated integrin complexes to form focal adhesions and stress fibers. Thus, recent studies on Rho effector molecules have enabled us to postulate the molecular mechanism of Rho-induced cell adhesion. Future studies will examine various hypotheses raised by such a model and test if a similar mechanism can be applied to other actin-based processes regulated by Rho.

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