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Activation of moesin and adducin by Rho-kinase downstream of Rho

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Abstract

The Rho GTPase (Rho) is a member of the Rho family, which belongs to the Ras superfamily of GTP-binding proteins. Like other GTP-binding proteins, Rho exists in two conformational states, an inactive GDP-bound form and an active GTP-bound form. Active Rho interacts with specific effectors to regulate the actin cytoskeleton and to mediate a variety of biological functions in cells. Rho-associated kinase (Rho-kinase) is the most studied Rho-effector, and studies of its biochemical and cell biological functions have provided us with useful information for understanding the molecular mechanisms of the actions of Rho. This review aims to summarize the roles of Rho and Rho-kinase in the regulation of the cytoskeletons.

Keywords: Rho; Rho-kinase; Adducin; ERM family proteins; Membrane ruffling; Microvilli

1. Introduction

Temporal and spatial reorganizations of the actin cytoskeleton occur upon stimulation by extracellular signals such as growth factors, which influence a wide variety of cellular functions including cell shape changes, cell motility, contraction, cell adhesion, and cytokinesis [1–3]. Accumulating evidence indicates that the small GTPase Rho is a key molecule in the reorganization of the actin cytoskeleton [4–6]. Rho, together with Rac, Cdc42, and TC10, belongs to the Rho family. Like all of the small GTPases, the activity of Rho in cells is regulated by the cycle between its inactive GDP- and active GTP-bound forms. The latter form of Rho interacts with specific effectors to exert cellular functions. Rho participates in signaling pathways that regulate actin cytoskeletal structures, such as stress fibers, and cell–substratum adhesions, such as focal adhesions, in fibroblasts [7]. Rho is also involved in the regulation of cell morphology [8], cell aggregation [9], cadherin-mediated cell–cell adhesion [10], cell motility [11,12], cytokinesis [13,14], membrane

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ruffling [15], neurite retraction [16,17], microvilli formation [18] and smooth muscle contraction [19,20]. Rho-kinase, which is a serine/threonine kinase identified as a specific effector of Rho [21–23], regulates the phosphorylation state of the myosin light chain (MLC) by the direct phosphorylation of MLC [24] and by the inactivation of myosin phosphatase through the phosphorylation of the myosin-binding subunit (MBS) [25]. The MLC phosphorylation confers contractility to actin–myosin filaments and thereby induces the formation of stress fibers and focal adhesions [26–28], smooth muscle contraction [29], and neurite retraction [30–32].

Actin filaments also interact with multiple proteins and indirectly with the plasma membrane, and together they constitute a specific membrane domain, the 'cell cortex'. In the cell cortex, the cortical cytoskeletons, such as the bundles of actin filaments or the meshworks of spectrin and actin filaments, are linked to the plasma membrane via a group of proteins called the bridge proteins. The formation of the cortical cytoskeleton and its linkage to the membrane are regulated during dynamic cellular processes. Current results indicate that Rho-kinase phosphorylates the ERM (ezrin/radixin/moesin) family proteins [33] and adducin [34], in addition to MLC and MBS. The ERM family proteins are known to serve as a 'bridge' between the plasma membrane and the actin cytoskeleton [35,36], and adducin is known to serve as an assembly factor for the spectrin–actin meshwork [38]. Phosphorylation of the ERM family proteins and adducin by Rho-kinase regulates the dynamics of the plasma membrane, such as microvilli formation [39] and membrane ruffling [40], respectively. The emphasis in this review is on the roles of Rho and Rho-kinase in the regulation of the cytoskeletons.

2. Rho-kinase as a specific effector of Rho

A large number of effectors of Rho have been identified by means of affinity chromatography, ligand overlay assays, or the yeast two-hybrid system. These effectors include Rho-kinase/ROK/ROCK [21–23], the myosin-binding subunit (MBS) of myosin phosphatase [25], protein kinase N (PKN)/PRK1 [41,42], rhophilin [42], rhotekin [43], citron [44], citron kinase [45], and p140 mDia [46]. Among these effectors, Rho-kinase/ROK/ROCK has been studied most extensively. Rho-kinase was identified as a GTP·Rho-binding protein from bovine brain by affinity column chromatography on matrix-bound GTP·Rho [22]. Rho-kinase was also identified as ROKα [21] and as ROCK2 [47]. ROKβ [26]/ROCK1 [23] is an isoform of Rho-kinase/ROKα/ROCK2. The structure of Rho-kinase is shown schematically in Fig. 1. Rho-kinase has a kinase domain in the NH_2 (N)-terminal portion, followed by a coiled-coil domain and a pleckstrin-homology (PH) domain. The kinase domain of Rho-kinase has 72% sequence homology with the kinase domain of myotonic dystrophy kinase [22]. The Rho-binding domain of Rho-kinase is localized in the carboxy (C)-terminal portion of the coiled-coil domain. The activity of Rho-kinase is enhanced by Rho-binding. Although the PH domain of Rho-kinase is expected to determine the localization of Rho-kinase, the exact functions of this domain have not been reported. Two substrates of Rho-kinase were first identified: MBS of myosin phosphatase and myosin light chain (MLC) [22,24,25]. Myosin phosphatase is composed of three subunits: MBS, a 37-kDa type 1 phosphatase catalytic subunit, and a 20-kDa regulatory subunit. Myosin phosphatase binds to phosphorylated MLC via MBS and dephosphorylates it. The C-terminal portion of MBS interacts with GTP-bound Rho [25]. The exact relevance of Rho-binding to MBS has not been elucidated, although activated Rho translocates MBS to the plasma membrane [25]. The phosphorylation of MBS by Rho-kinase leads to the inactivation of myosin phosphatase [25]. Phosphorylation of MLC and MBS by Rho-kinase, therefore, would induce the enhancement of MLC phosphorylation, leading to the activation of myosin ATPase and the assembly of myosin–actin filaments, as shown in Fig. 2. Consistent with this observation, the addition of dominant active Rho-kinase induces the formation of stress fibers through MLC phospho-

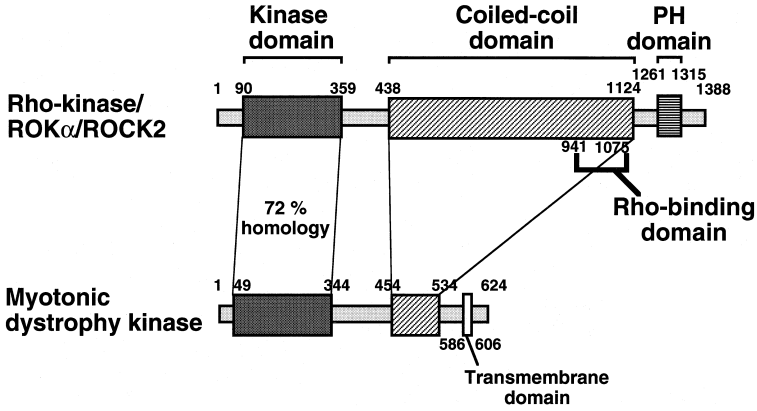

Fig. 1. Structures of Rho-kinase.

rylation in fibroblasts, whereas the addition of dominant negative Rho-kinase inhibits the LPA- or dominant active Rho-induced formation of stress fibers [26–28,48]. Also, the addition of dominant active Rho-kinase to permeabilized vascular smooth muscle induces contraction through MLC phosphorylation [29]. A specific chemical inhibitor for Rho-kinase selectively inhibits smooth muscle contraction by inhibiting GTP-dependent sensitization to suboptimal Ca^{2+}, and suppresses both hypertension in several hypertensive rat models [49] and vasospasm of the porcine coronary artery [50]. In neuronal cells, neurite retraction is also regulated by Rho-kinase through MLC phosphorylation [30–32]. Thus, the Rho-kinase-mediated increase of MLC phosphorylation may account for the mechanisms by which Rho regulates the formation of stress fibers and focal adhesions, smooth muscle contraction, and neurite retraction. More detailed information about other Rho-effectors can be found in previous reviews [4–6].

3. Identification of substrates of Rho-kinase other than MLC and MBS

We have searched for substrates of Rho-kinase

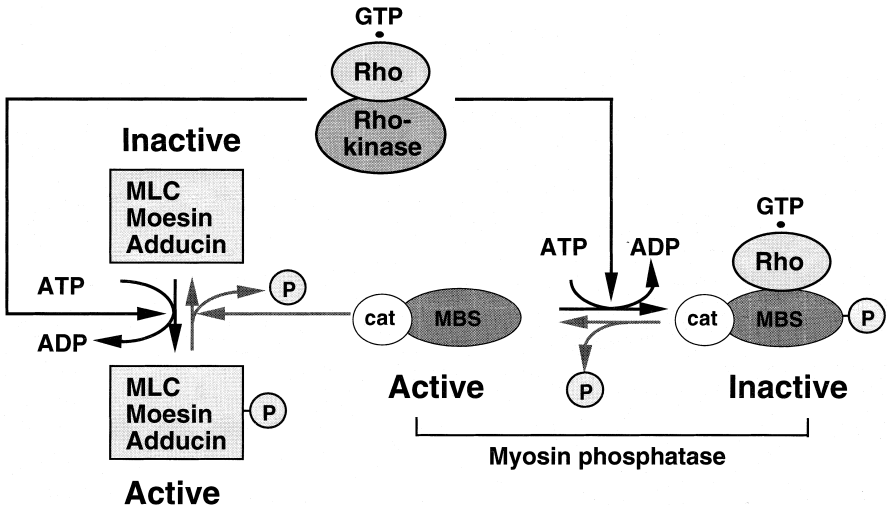

Fig. 2. Model for the regulation of the phosphorylation level of substrates by Rho-kinase and myosin phosphatase; cat, catalytic subunit of myosin phosphatase.

and found that ERM family proteins and adducin are good substrates both in vitro and in vivo (Table 1). By studying the function of the Rho-kinase-mediated phosphorylation of these substrates in intact cells, we have shown that Rho-kinase regulates a variety of cellular functions.

3.1. Moesin and microvilli formation

Actin filaments are linked to membrane integral proteins at the plasma membrane through certain bridge proteins. Although the association between the plasma membrane and the actin cytoskeleton may be dynamically regulated in cells during cell movement and the mitotic phase, the molecular mechanism behind the association has remained unclear. The ERM family comprises three closely related proteins (approx. 75% identity): ezrin; radixin; and moesin [51]. Their N-terminal domains ($\sim$ 300 aa) are highly conserved ($\sim$ 85% identity), and show sequence similarity to the N-terminal domain of band 4.1, which links the spectrin–actin meshwork to the plasma membrane protein glycophorine C [52]. The ERM family proteins are enriched at the actin-rich structures, such as cleavage furrows, microvilli, filopodia, and membrane ruffles, where they are thought to link the actin filaments with the plasma membrane. An antisense oligonucleotide analysis revealed that the ERM family proteins play a pivotal role in the formation of the microvilli, the cell–substratum and cell–cell adhesion [53]. The N-terminal and C-terminal domains of the ERM family proteins are thought to bind directly to some integral membrane proteins, such as CD44, CD43, and ICAM-1, and to actin filaments, respectively [35–37]. The native ERM proteins exist in either a dormant (inactive) or active state. In the dormant ERM family, an intramolecular association between the N- and C-terminal domains masks the membrane-association sites of the N-terminal domain and the F-actin-binding sites of the C-terminal domain. Activation of the ERM family proteins by signals is thought to lead to the exposure of the membrane-association site and the F-actin-binding site [35–37]. Recent evidence suggests that Rho regulates the association between CD44 and the ERM family proteins [54]. A permeable cell reconstitution assay showed that the ERM family proteins are essential for Rho- and Rac-induced cytoskeletal reorganization [55]. Rho might be involved in the regulation of the activity of the ERM family. In thrombin-activated platelets, moesin is phosphorylated at Thr^{558} [56]. This phosphorylation is associated with plasma membrane in macrophages and various tissues and is thought to be required for the stable interaction of moesin with actin [57,58]. Recently,

Table 1
Substrates of Rho-kinase

Substrates	Changes in functions	Biological properties
Myosin	Enhancement of binding of myosin to F-actin	Stress fiber formation Focal adhesion formation
MBS	Inhibition of myosin phosphatase	Smooth muscle contraction Neurite retraction
ERM (ezrin, radixin, moesin)	Activation of ERM	Microvilli formation
Adducin	Enhancement of binding to F-actin	Membrane ruffling cell motility
Intermediate filament (GFAP, vimentin)	Disassembly of filaments	Segregation of filaments in cytokinesis[a]

[a] See Yasui et al. [76].

we have found that Rho-kinase phosphorylates the ERM family proteins in vitro [33]. The site of phosphorylation by Rho-kinase is a conserved Thr residue in the C-terminus: $Thr^{567, 564, 558}$ of ezrin, radixin, and moesin, respectively. This phosphorylation inhibits the interaction between the N- and C-terminal domains of the ERM proteins in vitro [33]. This result suggests that the phosphorylation by Rho-kinase activates the ERM family proteins by releasing the intramolecular suppression, or may maintain the activity of the proteins by interfering with the intramolecular suppression.

As described above, the ERM family proteins are essential components of microvilli-like structures on polarized epithelial cells, fibroblasts, and lymphocytes. Studies have shown that LPA induces the relocalization of the ERM family proteins into microvilli-like structures in NIH3T3 cells, whereas *C. botulinum* C3 toxin inhibits this LPA-induced relocalization [18]. The expression of $RhoA^{V14}$ induces the formation of microvilli-like structures and the relocalization of the ERM family proteins into these structures in both Rat1 and NIH3T3 cells [18]. We have recently shown that the expression of $RhoA^{V14}$ in COS7 cells induces the phosphorylation of moesin at Thr^{558} and the formation of microvilli-like structures at the apical membranes, where Thr^{558}-phosphorylated moesin accumulates, whereas the expression of dominant negative Rho-kinase inhibits both of these processes [39]. The expression of dominant active Rho-kinase also induces moesin phosphorylation and the formation of microvilli-like structures. Moesin mutated at the site of phosphorylation by Rho-kinase to aspartate ($moesin^{T558D}$), which may mimic phosphorylated moesin, induces the formation of microvilli-like structures. Recently, similar results with mutated ezrin has been reported in A431 epithelial cells [59]. $Moesin^{T558A}$, which is not phosphorylated by Rho-kinase, inhibits the $RhoA^{V14}$-induced formation of microvilli-like structures [39]. Thus, Rho-kinase appears to regulate moesin phosphorylation downstream of Rho in vivo, and the phosphorylation of moesin by Rho-kinase seems to play a crucial role in the formation of microvilli-like structures. It is

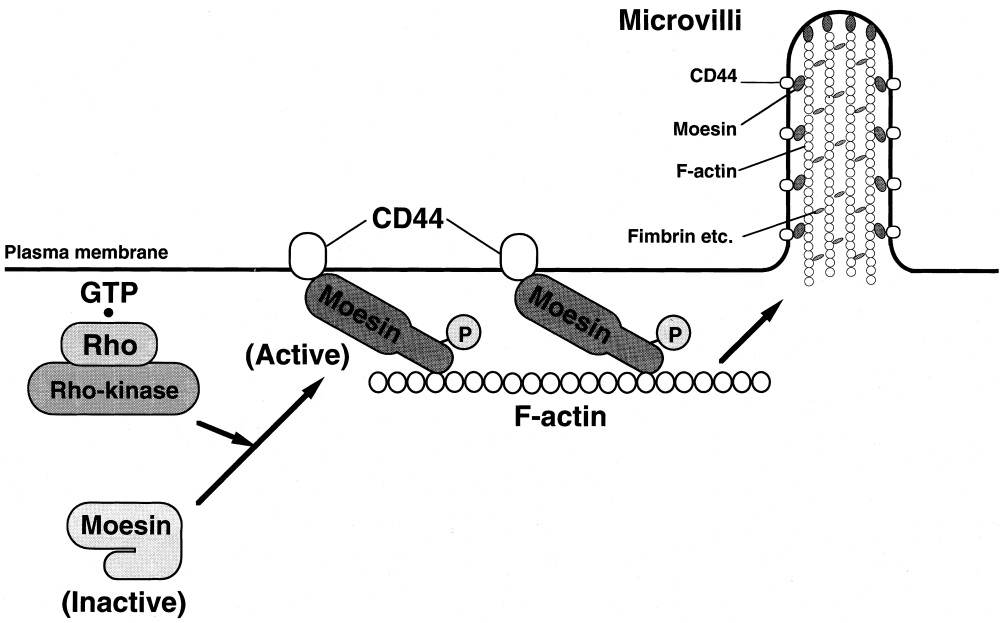

Fig. 3. Activation of moesin by a Rho/Rho-kinase pathway. In the resting state, moesin is inactivated via an intramolecular association between the N- and C-terminal domains. Activated Rho-kinase phosphorylates moesin, and releases the intramolecular association of moesin. Then, moesin can associate with the membrane binding partner and F-actin. A number of activated moesin proteins may lead to the formation of microvilli, together with an F-actin bundling protein, such as fimbrin.

likely that Rho-kinase increases the pool of activated ERM family proteins, and recruits the proteins to the apical surface of the cell. As a result, the activated ERM proteins on the apical surface may promote the association of the plasma membrane with the actin cytoskeleton, where in turn the formation of the microvilli may be induced (Fig. 3).

3.2. Adducin and membrane ruffling

Membrane ruffling, which is a dynamic three-dimensional movement of the plasma membrane, is rapidly induced in cells in response to certain extracellular signals, and is also seen in the leading edges of motile cells [3,60]. Membrane ruffles contain an abundance of complicated cytoskeletal structures composed of F-actin and F-actin associated proteins [3]. Although the formation of membrane ruffles is tightly coupled to actin polymerization and depolymerization beneath the plasma membrane, the molecular mechanism of membrane ruffling is not fully understood. Rac regulates membrane ruffling through actin polymerization [5]. In addition to Rac, Rho is also thought to regulate the membrane ruffling downstream of extracellular signals in certain types of cells, because C3 inhibits the TPA or HGF-induced membrane ruffling in epithelial cells, such as MDCK and KB cells [15,61]. TPA or HGF stimulates the motility of epithelial cells, by initially inducing a centrifugal spreading of cell colonies, followed by a disruption of cell–cell adhesions and then cell scattering, accompanied by membrane ruffling. Rho also plays a crucial role in the HGF or TPA-induced motility of keratinocytes (308R cells) [12]. However, it has not yet been elucidated how Rho regulates either membrane ruffling or cell motility. Recently, we have found that the expression of a dominant negative Rho-kinase inhibits the HGF or TPA-induced membrane ruffling and the wound-induced cell migration, indicating that Rho-kinase is involved in membrane ruffling and cell motility [40].

We have found that Rho-kinase phosphorylates adducin in vitro [34]. Adducin was originally isolated from the erythrocyte membrane skeleton as a calmodulin-binding protein [38]. Adducin is expressed in many cells and tissues as well as erythrocytes. It consists of two subunits: either α and β or α and γ [62,63]. Adducin binds to F-actin and the spectrin–F-actin complex, and subsequently promotes the binding of spectrin to the spectrin–F-actin complex [38]. Adducin also bundles [64] or caps actin filaments [65]. Adducin is thought to regulate the organization of the spectrin–F-actin meshwork beneath the plasma membrane. Adducin is also phosphorylated by protein kinase C (PKC) and protein kinase A (PKA) [63,66]. The phosphorylation of adducin by PKA and PKC reduces the abilities of adducin to associate with F-actin and the spectrin–F-actin complexes and to recruit spectrin to F-actin [67,68]. On the other hand, the phosphorylation of adducin by Rho-kinase enhances its F-actin-binding activity [34]. Recently, we have identified the sites of phosphorylation of α-adducin by Rho-kinase as Thr^{445} and Thr^{480} [40]. Rho-kinase phosphorylates α-adducin at Thr^{445} in the membrane ruffling area of MDCK cells during the action of TPA or HGF [40]. The expression of α-$adducin^{T445A,\ T480A}$ (substitution of Thr residues by Ala), which is not phosphorylated by Rho-kinase, inhibits TPA-induced membrane ruffling without inhibiting the phosphorylation of other substrates, such as MLC and moesin. The expression of α-$adducin^{T445D,\ T480D}$ (substitution of Thr residues by Asp), which may mimic α-adducin phosphorylated by Rho-kinase, counteracts the inhibitory effect of the dominant negative Rho-kinase on TPA-induced membrane ruffling. These results indicate that the phosphorylation of adducin by Rho-kinase is necessary for membrane ruffling. The expression of either C3 and dominant negative Rho-kinase or α-$adducin^{T445A,\ T480A}$ in NRK fibroblasts inhibits the membrane ruffling, and thereby inhibits the wound-induced cell migration [40]. Taken together, these results suggest that α-adducin is one of the substrates for Rho-kinase involved in cell motility, probably by regulating the membrane ruffling in the leading edges of motile cells. α-Adducin phosphorylated by the Rho/Rho-kinase pathway may promote the assembly of a spectrin–F-actin meshwork in the membrane ruffles.

MLC is also thought to play an important role

in cell motility. The injection of an anti-MLC kinase antibody diminishes the cell motility of macrophages [69]. Moreover, phosphorylated MLC is enriched in both the leading and rear ends of motile fibroblasts and epithelial cells [70], suggesting that a force derived from the myosin–actin filament, driven by the MLC phosphorylation, contributes to cell motility. Thus, Rho, acting through Rho-kinase, appears to regulate cell motility through the spatial and temporal regulation of phosphorylation of certain substrates, including adducin and MLC.

3.3. Interaction of MBS with substrates of Rho-kinase

Myosin phosphatase forms a complex with MLC through MBS. Rho-kinase not only directly phosphorylates MLC, but also inactivates myosin phosphatase through the phosphorylation of MBS [25]. As a result, the level of MLC phosphorylation is elevated. Here, we have found that other substrates of Rho-kinase, such as moesin and adducin, are also MBS-binding partners [34,71]. Myosin phosphatase associates with moesin or adducin through MBS, and dephosphorylates the moesin or adducin that have been phosphorylated by Rho-kinase [34,71]. The phosphatase activity of myosin phosphatase toward moesin or adducin is inhibited by the phosphorylation of MBS by Rho-kinase [34,71]. These observations suggest that the phosphorylation states of certain substrates can be tightly regulated by Rho-kinase and myosin phosphatase when Rho is transiently activated (Fig. 2). Recently, the Na^+–H^+ exchanger, which is involved in the formation of stress fibers and focal adhesions downstream of Rho [72], was shown to be phosphorylated by Rho-kinase in the C-terminal portions, and then to be activated [73]. We have previously found that Rho-kinase also phosphorylates intermediate filaments, such as glial fibrillary acidic proteins (GFAP) and vimentin, and thereby regulates the disassembly of these filaments [74,75]. It remains to be clarified whether GFAP, vimentin, and/or the Na^+–H^+ exchanger bind to MBS and are dephosphorylated by myosin phosphatase.

4. Conclusion and perspectives

Although it is well recognized that Rho regulates various cellular functions, until recently the molecular mechanisms behind this regulation have not been well understood. Studies in recent years have focused on the identification and characterization of specific effectors of Rho. Now, it is recognized that Rho, together with its effector, Rho-kinase/ROCK/ROK, mediates a diverse array of cellular events. Rho-kinase phosphorylates MLC, which is involved in the formation of stress fibers and focal adhesions, smooth muscle contraction, and neurite retraction. Rho-kinase also phosphorylates the ERM family proteins, which are involved in the formation of microvilli, and phosphorylates adducin, which participates in membrane ruffling and cell motility. Furthermore, Rho may tightly regulate the phosphorylation levels of these substrates through the interactions of both Rho-kinase and the MBS of myosin phosphatase, and this may be important for the temporal and spatial regulation of the function of these substrates in response to extra/intracellular signals. The identification of additional substrates of Rho-kinase may help us to better understand the signaling pathways downstream of Rho, and the unidentified Rho functions.

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