Rho-kinase in human neutrophils: a role in signalling for myosin light chain phosphorylation and cell migration

Verena Niggli*

Department of Pathology, University of Bern, Murtenstr. 31, P.O. Box 62, CH-3010 Bern, Switzerland

Received 24 November 1998; received in revised form 15 January 1999

Abstract The role of a Rho-associated coiled-coil forming kinase in migration of neutrophils has been investigated. Rho-associated coiled-coil forming kinase I was expressed in human neutrophils. Chemotactic peptide led to a Rho-associated coiled-coil forming kinase-dependent increase in phosphorylation of myosin light chain. This was determined with the help of an antibody directed against serine 19-phosphorylated myosin light chain and an inhibitor of Rho-associated coiled-coil forming kinase (Y-27632). Y-27632 suppressed myosin light chain phosphorylation and chemotactic peptide-induced development of cell polarity and locomotion with similar potency (ED_{50} 0.5–1.1 μM). The data strongly suggest that a Rho-associated coiled-coil forming kinase isoform, activated in human neutrophils exposed to chemotactic peptide, is important for motile functions of these cells.

Key words: Rho-associated coiled-coil forming kinase; Cell migration; Myosin light chain; Signal transduction; Human neutrophil

1. Introduction

In order to efficiently reach sites of infection and destroy invading bacteria, neutrophils are capable of rapid directed movement along a chemotactic gradient. This directed movement is dependent on a functional cytoskeleton [1]. Directed cell locomotion requires, in addition to a dynamic actin network, development of a polarized shape and reversible contacts with the substratum. How these events are regulated is still poorly understood. Investigations have been hampered by the fact that neutrophils, as terminally differentiated cells, are not amenable to genetic manipulations.

Recent studies have revealed a complex network of signalling systems in neutrophils [2]. The available evidence suggests an important role of the small GTP-binding protein Rho in neutrophil migration and adhesion. Exoenzyme C3, introduced by electroporation into bovine neutrophils, inactivates Rho selectively and suppresses cell migration [3]. It could also be shown that Rho is crucial for integrin-mediated adhesion of neutrophils to fibrinogen using the same technique [4]. However the targets of Rho mediating these effects have not yet been defined. The Rho-associated coiled-coil forming kinases (ROCKs) ROCK I (= p160ROCK) and ROCK II, closely related isoforms, are known to be activated by association with GTP-Rho [5]. These enzymes have been shown to be involved in formation of focal contacts and stress fibers in several cell types, acting possibly by affecting myosin light chain (MLC) phosphorylation [5] or by phosphorylating ezrin/radixin/moesin proteins [6]. A recently developed compound, Y-27632, suppresses ROCK I and II activity in vitro with a K_i of 0.14–1.0 μM. Other enzymes, such as protein kinase C, the cAMP-dependent kinase or MLC-kinase, are inhibited at 50–>1700-fold higher concentrations. Y-27632 is cell permeable and inhibits Rho-induced formation of focal adhesions and stress fibers in HeLa-cells [7]. I now present new data on the presence of ROCK I in human neutrophils, on its role in MLC phosphorylation and in modulating neutrophil migration.

2. Materials and methods

2.1. Reagents and antibodies

fNLPNTL was from Bachem, Bubendorf, Switzerland, HSA was from Behringwerke, Marburg, Germany, Pefabloc was from Boehringer Mannheim, Rotkreuz, Switzerland and protease inhibitor cocktail for mammalian cell extracts was from Sigma. Y-27632 was kindly provided by Yoshitomi Research Laboratories, Japan. Stock solutions of Y-27632 (10 mM) were prepared in H_2O and aliquots were stored at −20°C. A polyclonal antibody raised in rabbits against amino acids 2–15 of p160 ROCK (antiserum 20490 [8]) was kindly provided by Dr. T. Ishizaki. A polyclonal antibody raised in rabbits against MLC phosphorylated on serine 19 (antiserum pp2b [9]) was a kind gift from Dr. F. Matsumura.

2.2. Isolation of human neutrophils

Neutrophils were isolated from heparinized human blood as detailed in [10]. Isolated neutrophils were resuspended in a medium containing 138 mM NaCl, 6 mM KCl, 1 mM Na_2HPO_4, 5 mM $NaHCO_3$, 5.5 mM glucose, 20 mM HEPES, pH 7.3. Divalent cations and HSA were added for locomotion assays (see below). Approximately 95% of the leukocytes were neutrophils, with 0.2–0.4 platelets per neutrophil.

2.3. Immunodetection of ROCK I and of serine-19 phosphorylated MLC

For detection of ROCK I, neutrophils were treated with diisopropylfluorophosphate in order to block endogenous proteases, followed by solubilization of cells in 2% SDS, 10 mM Tris-Cl, pH 7.4, 10 mM EDTA, 4 mM Pefabloc, 25 μl protease inhibitor cocktail/ml, and protein determination of cell lysates as described [11]. Lysates were applied to SDS polyacrylamide gradient gels and transblotted to nitrocellulose [11]. Blots were exposed to a polyclonal anti-ROCK I antibody (diluted 1:500). For visualization of bound antibody, a goat anti-rabbit IgG antibody, conjugated to horseradish peroxidase (Bio-Rad), diluted 1:10 000 was used, followed by ECL detection. As a positive control for ROCK I immunoreactivity, human platelets were isolated as described [11], solubilized in the same buffer as neutrophils (see above) and subjected to immunoblotting.

For assays of the effect of cell stimulation on MLC phosphorylation, neutrophils (5×10^6 cells/ml) were exposed to stimuli and inhibitor, as indicated in Section 3. They were then centrifuged at $380\times g$ to terminate the reaction, solubilized immediately in a buffer contain-

*Fax: (41) (31) 381 34 12.
E-mail: niggli@patho.unibe.ch

Abbreviations: fNLPNTL, *N*-formyl-L-norleucyl-L-leucyl-L-phenylalanyl-L-norleucyl-L-tyrosyl-L-lysine; HSA, human serum albumin; MLC, myosin light chain; ROCK, Rho-associated coiled-coil forming kinase

PII: S0014-5793(99)00098-8

ing 1% SDS, 50 mM DTT, 15% (v/v) glycerol, 62.5 mM Tris, pH 6.8, 0.001% bromophenol blue and subjected to immunoblotting using a polyclonal antibody directed against the serine-19 phosphorylated MLC, diluted 1:500.

2.4. Analysis of shape changes and localization of F-actin

Neutrophils ($3–6\times10^6$ cells/ml) were incubated in medium in a reciprocating waterbath at 37°C without or with Y-27632 and fNLPNTL, as indicated in Section 3. The reaction was stopped by fixing the cells in 1% glutaraldehyde (final concentration) for 30 min. Cell shape was determined as described [12]. The concentration-dependent effects of Y-27632 were not affected by the cell density in the range of $3–6\times10^6$ cells/ml. For visualization of F-actin, cells were fixed with paraformaldehyde and stained with rhodamine-phalloidin as described [10].

2.5. Cell locomotion

For locomotion assays, a discontinuous Ficoll-metrizoate gradient instead of neutrophil isolation medium was used for removal of mononuclear cells [10]. Neutrophils (3×10^6/ml) were incubated in medium containing in addition 1.1 mM $CaCl_2$, 1 mM $MgCl_2$, 100 μM EGTA, 3% HSA and inhibitor and/or stimulus at 37°C in a reciprocating waterbath. The cells were centrifuged at $300\times g$ for 5 min and resuspended in the same medium. Subsequently, the % of migrating cells and the mean speed of migration was determined as described [10].

2.6. Statistical analysis of data

The data correspond to mean ± S.E.M. of n independent experiments. Differences between the data were analyzed with the Student's t-test for paired data, with a P value of <0.05 considered significant.

3. Results

3.1. ROCK I is present in human neutrophils

A polyclonal antibody selective for ROCK I [8] reacted with a major band of 158 kDa in human neutrophil lysates, comigrating with a band in human platelets (Fig. 1). Human platelets have previously been shown to contain ROCK I [8], and cloning and sequencing of ROCK I has revealed a calculated molecular mass of 158 166 Da [13]. Quantitative evaluation of the immunoblots by scanning shows, that neutrophils contain approximately eight-fold less ROCK I, relative to the total amount of protein, than platelets. ROCK I detected in neutrophil lysates was not due to contamination of the preparation with platelets, as a transblot of the corresponding amount (1 μg) of platelet protein present in 150 μg neutrophil protein for the preparation shown in Fig. 1 did not exhibit a detectable ROCK I band (data not shown).

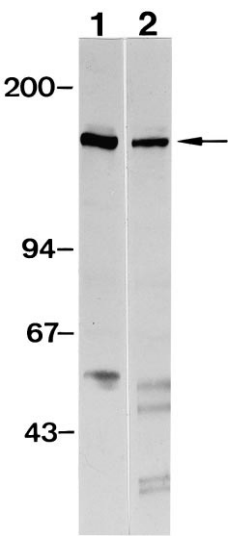

Fig. 1. Immunodetection of ROCK I in human neutrophils and platelets. Total platelet proteins (50 μg, lane 1) and total neutrophil proteins (150 μg, lane 2) were subjected to electrophoresis through a 5–10% gradient gel, followed by transfer to nitrocellulose and immunodetection of ROCK I. The polyclonal antibody reacts with a major band of 158 000 Da in both platelets and neutrophils (arrow). Numbers at the left indicate molecular masses of standard proteins in kDa.

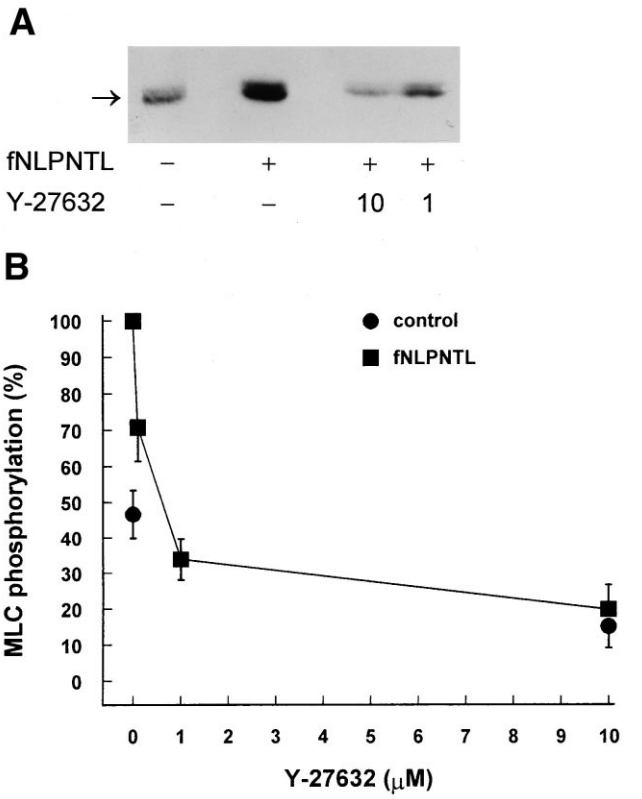

Fig. 2. The ROCK inhibitor Y-27632 suppresses basal and chemotactic peptide-induced increases in phosphorylation of MLC at serine-19 in neutrophils. A: Cells were preincubated in medium with or without 1 μM (1) or 10 μM (10) Y-27632 at 37°C for 1 h, followed by addition of medium (−) or 1 nM fNLPNTL (+), as indicated, and a further incubation for 30 min. Cell lysates were then analyzed by immunoblotting using an antibody specific for MLC phosphorylated on serine-19. The arrow indicates the main band reacting with the antibody against MLC phosphorylated on serine-19. B: Quantitative evaluation of the effects of Y-27632 on MLC phosphorylation in resting and stimulated cells. Cells were pretreated without or with increasing amounts of Y-27632, as described for A, followed by the addition of medium (control) or 1 nM fNLPNTL, and a further incubation for 30 min. Cells were then analyzed by immunoblotting and scanning of the MLC band. Mean ± S.E.M. of 3–6 experiments.

3.2. A ROCK-selective inhibitor suppresses chemotactic peptide-induced increases in phosphorylation of MLC on serine-19

As shown previously, ROCK isoenzymes stimulate incorporation of phosphate into the regulatory MLC II, by inhibiting myosin phosphatase [14] and by directly phosphorylating serine-19 of MLC [15]. In order to study MLC phosphorylation, an antibody specifically reacting with a phosphopeptide corresponding to serine-19-phosphorylated MLC [10] has been used. This antibody allows a selective assessment of MLC

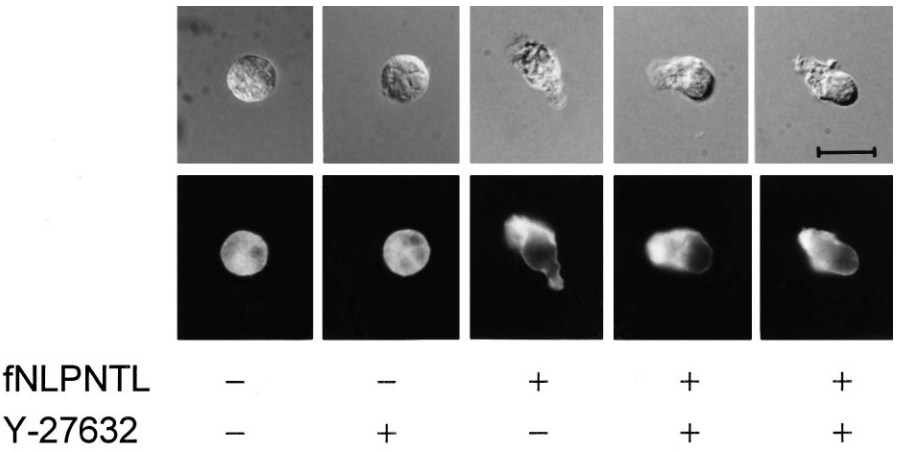

Fig. 3. Y-27632 prevents fNLPNTL-induced development of polarity in human neutrophils. Cells were preincubated in medium without (−) or with (+) 10 μM Y-27632 at 37°C for 1 h, followed by addition of medium (−) or 1 nM fNLPNTL (+) as indicated and a further incubation for 30 min. At the end of the incubation period, cells were fixed and stained with rhodamine-phalloidin to visualize F-actin. Photographs were obtained with Nomarski optics (upper row of panels) or in fluorescent light (lower row of panels). For cells preincubated with 10 μM Y-27632 follwed by addition of fNLPNTL, two typical examples of cells are shown. Bar: 10 μm.

phosphorylation mediated by ROCK I and MLC-kinase. It reacted in neutrophil lysates with a main band of approximately 20 kDa. As shown in Fig. 2A, some background phosphorylation of MLC was already detectable in resting cells. Treatment of neutrophils with 1 nM fNLPNTL for 30 min resulted in a 2.4 ± 0.4-fold ($n = 6$, $P < 0.0005$) increase in serine-19-phosphorylated MLC (Fig. 2A,B). Pretreatment of neutrophils with 10 μM Y-27632, an inhibitor with selectivity for ROCK [7], prior to addition of chemotactic peptide resulted in almost complete suppression of MLC phosphorylation on serine-19 (80 ± 7% inhibition, $n = 4$, Fig. 2A,B). Phosphorylation in control cells was also markedly inhibited by 68 ± 8% ($n = 3$, Fig. 2B). Half-maximal effects on MLC phosphorylation in stimulated cells were obtained at approximately 0.5 μM Y-27632 (Fig. 2B).

3.3. Y-27632 suppresses chemotactic peptide induced shape changes and locomotion in human neutrophils

As myosin has been implicated to play a crucial role in cell migration, the effect of suppressing MLC phosphorylation with Y-27632 on shape changes, F-actin localization and migration of neutrophils was investigated. Treatment of resting human neutrophils for 1 h with 10 μM Y27632 did not affect the smooth, spherical shape and diffuse F-actin localization as visualized by rhodamine phalloidin, which is exhibited by 80–90% of these cells (Fig. 3). Human neutrophils, exposed to low (nM) concentrations of chemotactic peptide in suspension initially form ruffles all over the cell body. They subsequently adopt an elongated polarized shape with a contracted tail and a broad, F-actin-rich front lamellae (Fig. 3). Preincubation of human neutrophils with 10 μM Y-27632 did not affect the first rapid phase of ruffling (data not shown) but it clearly suppressed tail contraction occurring later. Cells became less elongated and more spherical, non-polar with F-actin-rich protrusions on one side (Fig. 3). Half-maximal suppression of development of polarity was obtained in the range of 0.8–1.1 μM in three experiments. Development of polarity was maximally inhibited in the range of 4–10 μM Y-27632 by 83 ± 6% (mean ± S.D., $n = 3$) (Fig. 4).

I investigated whether cells treated with Y-27632 and lacking a contracted tail were able to migrate. The effect of 10 μM Y-27632 on fNLPNTL-stimulated locomotion of neutrophils in the absence of a gradient (= chemokinesis) was investigated. As shown in Table 1, the p160ROCK inhibitor indeed suppressed chemokinesis to the same extent as development of polarity (78 ± 10% inhibition by 10 μM Y-27632, $n = 4$). Moreover, the mean speed of the remaining locomoting cells in the presence of 10 μM Y-27632 was significantly ($P < 0.025$) reduced compared to untreated controls (Table 1).

Interference with actin reorganization could contribute to suppression of cell migration. Therefore the effect of Y-27632 on actin reorganization in neutrophils was assessed. However, preincubation of neutrophils with 10 μM Y-27632 did not prevent increases in cytoskeletal actin induced by incubation with 1 nM fNLPNTL for 1 or 30 min, determined as described [10]. In agreement with these results, neutrophils treated with Y-27632 prior to addition of the stimulus still formed F-actin-rich ruffles (Fig. 3).

Table 1
Y-27632 suppresses locomotion of human neutrophils

Addition	Migrating cells (%)	Speed of migration (μm/min)
Medium	0 ± 0	–
fNLPNTL (1 nM)	49 ± 6	5.3 ± 0.4
Y-27632 (10 μM) and fNLPNTL (1 nM)	12 ± 5	2.4 ± 0.3

Neutrophils (3×10^6/ml) were preincubated for 10 min without or with 10 μM Y-27632 at 37°C, followed by addition of medium or 1 nM fNLPNTL and a further incubation for 30 min. Subsequently the % of migrating cells and the mean speed of migrating cells was determined in slide coverslip preparations at 37°C using video microscopy. Mean ± S.E.M. of four independent experiments.

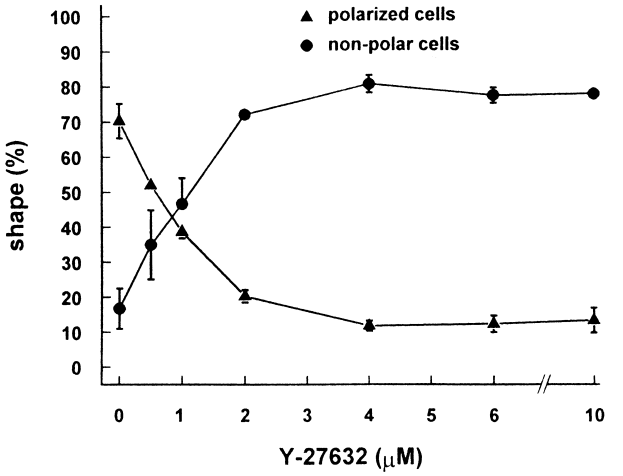

Fig. 4. Quantitative evaluation of the effect of Y-27632 on development of cell polarity. Cells were preincubated in the presence of increasing concentrations of Y-27632 for 1 h at 37°C, followed by the addition of 1 nM fNLPNTL and a further incubation for 30 min. Mean ± S.D. of three independent experiments (except for 0.5 μM: $n = 2$). (●) non-polar cells with surface projections; (▲) polarized cells with contracted tail.

4. Discussion

The Rho family of GTP-binding proteins has been implicated in signal transduction from membrane receptors to the cytoskeleton [16]! I now provide new data on the involvement of a downstream target of Rho, the Rho-activated kinase ROCK, in controlling development of polarity and migration of human neutrophils possibly via regulating MLC phosphorylation.

The isoform ROCK I is shown to be present in human neutrophils (Fig. 1). As a measure for the activity of ROCK, phosphorylation of MLC on serine-19 was assessed using a specific antibody. This site is phosphorylated by MLC-kinase and ROCK, but not by protein kinase C [15]. First, our results suggest that ROCK is constitutively active in resting cells, maintaining part of the basal level of MLC phosphorylation which does not result in shape changes (Figs. 2 and 3). Second, ROCK is also crucial for regulating MLC phosphorylation in stimulated cells. Chemotactic peptide induced a significant 1.5–3-fold increase in MLC phosphorylation on serine-19 in neutrophils, sustained for at least 30 min after addition of a stimulus, in agreement with previous data on overall MLC phosphorylation obtained by labelling of neutrophils with [^{32}P]orthophosphate [17]. Serine-19 phosphorylation was almost completely suppressed by 10 μM of the ROCK inhibitor Y-27632 (Fig. 2). MLC-kinase is not affected by Y-27632 under our conditions, as Y-27632 inhibits MLC-kinase with a $K_i > 250$ μM [7]. Y-27632 suppressed MLC phosphorylation in stimulated neutrophils with an ED_{50} of approximately 0.5 μM, in good agreement with the K_i of 0.14–1.0 μM obtained for inhibition of ROCK I purified from COS-cells and human platelets and the ED_{50} of 0.7 μM observed for inhibition of contraction of rabbit aortic strips [7]. Third, ROCK activity appears to be required for development of a polar shape with a contracted tail and for stimulated migration. In contrast to reports on other cell types, ROCK activity may not be crucial for the receptor-linked actin reorganization in neutrophils.

Neutrophils can migrate on certain substrates such as albumin-coated glass in the absence of a marked increase in intracellular Ca^{2+} [18]. In this context, it is of interest that in smooth muscle cells ROCK mediates contraction in the absence of increased Ca^{2+}. In permeabilized artery preparations for example, contraction can be induced either by addition of 1 μM free Ca^{2+} or GTPγS, but only the latter event can be suppressed by Y-27632 [7]. Likewise, ROCK could contribute to migration of neutrophils in the absence of increases in cytosolic Ca^{2+}.

In summary, evidence is provided for an important role of ROCK in controlling the tail contraction and locomotion of human neutrophils, possibly by regulating MLC phosphorylation. Other, as yet unidentified ROCK substrates, may also be involved in modulating the neutrophil migration downstream of ROCK. Future studies will focus on identifying such targets of ROCK in human neutrophils. Interesting candidates are for example proteins of the ezrin/moesin/radixin family [6], putative actin-membrane linkers.

Acknowledgements: This work was supported by the Swiss National Foundation for Scientific Research (Grant No. 31-49197.96). I thank Kathy Mujynya-Ludunge for the excellent technical assistance, Dr. E. Sigel for critical reading of the manuscript and Prof. H.U. Keller for the continuous support. I am grateful to Dr. T Ishizaki for providing the polyclonal anti-p160ROCK antibody and to Dr. F. Matsumura for the kind gift of the anti-serine-19-phosphorylated MLC antibody.

References

[1] Zigmond, S.H. (1993) Cell Motil. Cytoskelet. 25, 309–316.
[2] Bokoch, G.M. (1995) Blood 86, 1649–1660.
[3] Stasia, M.-J., Jouan, A., Bourmeyster, N., Boquet, P. and Vignais, P.V. (1991) Biochem. Biophys. Res. Commun. 180, 615–622.
[4] Laudanna, C., Campbell, J.J. and Butcher, E.C. (1996) Science 271, 981–983.
[5] Narumiya, S., Ishizaki, T. and Watanabe, N. (1997) FEBS Lett. 410, 68–72.
[6] Matsui, T., Maeda, M., Doi, Y., Yonemura, S., Amano, M., Kaibuchi, K., Tsukita, Sa. and Tsukita, Sh. (1998) J. Cell Biol. 140, 647–657.
[7] Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Maekawa, M. and Narumiya, S. (1997) Nature 389, 990–994.
[8] Fujita, A., Saito, Y., Ishizaki, T., Maekawa, M., Fujisawa, K., Ushikubi, F. and Narumiya, S. (1997) Biochem. J. 328, 769–775.
[9] Matsumura, F., Ono, S., Yamakita, Y., Totsukawa, G. and Yamashiro, S. (1998) J. Cell Biol. 140, 119–129.
[10] Niggli, V. and Keller, H.U. (1997) Eur. J. Pharmacol. 335, 43–52.
[11] Niggli, V. and Jenni, V. (1989) Eur. J. Cell Biol. 49, 366–372.
[12] Keller, H.U. and Niggli, V. (1994) J. Cell. Physiol. 161, 526–536.
[13] Ishizaki, T., Maekawa, M., Fujisawa, K., Okawa, K., Iwamatsu, A., Fujita, A., Watanabe, N., Saito, Y., Kakizuka, A., Morii, N. and Narumiya, S. (1996) EMBO J. 15, 1885–1893.
[14] Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A. and Kaibuchi, K. (1996) Science 273, 245–248.
[15] Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y. and Kaibuchi, K. (1996) J. Biol. Chem. 271, 20246–20249.
[16] Machesky, L.M. and Hall, A. (1996) Trends Cell Biol. 6, 304–310.
[17] Fechheimer, M. and Zigmond, S.H. (1983) Cell Motil. 3, 349–361.
[18] Maxfield, F.R. (1992) Trends Cell Biol. 3, 386–391.