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CELL BIOPHYSICS

Caldesmon Affects Actin Organization at the Leading Edge and Inhibits Cell Migration

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Abstract—The effect of the suppression of expression of the actin-binding protein caldesmon on the motility of nonmuscle cells has been studied. A more than a fivefold decrease in the content of this protein in cells by RNA interference led to the disturbance of the formation of actin stress fibers and acceleration of cell migration to the zone of injury of the monolayer. A stimulation of stationary cells by serum induced more than 1,5-fold accumulation of stress fibers only in control cells, but not in caldesmon-deficient cells. Similarly, the accumulation of actin filaments was observed in actively migrating cells of only wild type, but not in the cells with low caldesmon content. These changes occurred mainly at the leading edge of the migrating cell where the distinct structure of actin filaments was not seen in the absence of caldesmon. It was assumed that caldesmon inhibits cell migration due to the stabilization of actin in filaments and a decrease in the dynamics of monomeric actin at the leading edge of the migrating cell.

Key words: caldesmon, RNA interference, cell migration, actin filaments, leading edge of the cell

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Directed cell migration is a key event in embryogenesis, progenitor cell homing, growth of vessels and nerve fibres, inflammatory response and reparation of tissue injuries [1]. The direction of migration is determined by chemoattractant gradient (growth factors, cytokines and other extracellular stimuli), localization of their receptors at the leading edge of the cell, and also by existence of free space, as it happens during cell migration to a wound or a scratch at the cell monolayer.

The action of chemoattractants is realized through the variety of common intracellular mechanisms, which are not completely understood. To migrate, the cell undergoes a cyclic process of protrusion formation at the leading edge, protrusion attachment to substrate, cytoplasmic influx into them, detachment and tail retraction by cytoskeleton contraction [2]. It is generally accepted that it is the dynamics of cell leading edge that contributes to migration rate for the most part. Signal from chemoattractant, bound to the receptor, is transduced by cascade pathways to the proteins, which regulate actin polymerization at primary adhesion complexes of lamellopodium, the narrow leading strip at the edge of foliate lamella.

The growth of actin filaments towards the cell center is accompanied by the outward membrane growth to the opposite side and protrusion formation [3]. Pool of monomeric actin in lamella is made up by depolymerization of free ends of actin fibers under the action of cofilin, providing necessary recirculation of actin [4, 5].

The protrusion activity of lamellopodia depends on the number of focal adhesion contacts, concentration and accessibility of actin monomers, and also on the rate of assembling/disassembling of actin fibers [6]. All of these factors are regulated by actin-binding proteins. At the same time the activity of these proteins is under receptor control. Increasing of the number of actin polymerization sites and of forming actin fibers is provided by nucleating activity of formins and branching activity of Arp2/3 [7, 8]. Regulation of monomeric actin pool results from alteration of cofilin depolymerizing activity and also from profilin activity that blocks monomeric actin [9].

Proteins that mediate polymerization, depolymerization and actin fiber stability, represent a separate and heterogeneous group [10]. Generally, they are capping or laterally interacting proteins that alter polymerization rate, accessibility to cofilin. They also combine actin fibers into depolymerization-resistant stress-fibers or actin network. The multifunctional protein caldesmon is one of them. It interacts with actin, myosin and tropomyosin, and is also targeted by several receptor-activated cascades that regulate its activity [11]. In addition to stabilization of polymeric actin and alteration of its

Abbreviations: FCS, fetal calf serum; shRNA, short hairpin RNA; GFP, green fluorescent protein; PBS, phosphate buffered saline; CPFB, cytoskeleton protein fixation buffer; GAPDH, glyceraldehyde phosphate dehydrogenase.

mechanical properties [12], caldesmon inhibits actomyosin ATPase, therefore it may inhibit the tail retraction during cell movement.

Caldesmon is thought to be involved in regulation of cell migration [13, 14]. However, detailed mechanisms of its activity remain unclear. Caldesmon has been found to affect strength of cell adhesion [15], podosome formation [16], organization of cortical cytoskeleton [17] and stability of actin fibers [18]. It also regulates activity of the other actin-binding proteins, such as gelsolin and tropomyosin [19], fascin [20], Arp2/3 [21], which indirectly alter dynamics of intracellular actin. At the same time, the role of caldesmon in regulating the rate of cell movement has not been yet demonstrated.

Intracellular activity of caldesmon is determined by its binding to actin, in turn regulated by phosphorylation [22, 23]. We and others earlier found that increased chemotaxis is associated with MAP-kinase activation and caldesmon phosphorylation [17, 24], which reduces its binding to cytoskeleton [25]. Phosphorylation also regulates caldesmon activity in podosomes [26], cortical cytoskeleton [27], and stress-fibers [14].

Because phosphorylation inhibits the critical activity of caldesmon, we anticipated that knocking down caldesmon expression in cells would functionally reproduce its effects. Therefore, here we investigated the effect of decreased caldesmon expression on cell motility and state of actin cytoskeleton at the leading edge of migrating cells.

MATERIALS AND METHODS

Suppression of caldesmon expression by RNA-interference in HeLa 1469 cells. Human HeLa 1469 linear epithelial cells were cultured at 37°C and 5% CO_2 in DMEM medium, containing 10% fetal calf serum (FCS) (Hyclone, USA), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin.

To produce cells with stably decreased caldesmon expression the HeLa 1469 cells were transduced with lentiviral particles, bearing construct pSIH-CopGFP-HlshRNA (SBI, California) with inserted sequence of interfering RNA (shRNA) to the region of caldesmon gene (or luciferase gene in the case of control construct) and the sequence of green fluorescent protein (GFP) under the regulation of the common promoter. The appearance of GFP fluorescence in the transduced cell indicated the expression of the corresponding shRNA in this cell. The transduction efficiency was evaluated by flow cytometry using GFP fluorescence.

Caldesmon expression level in cells was measured by immunoblotting with antibody recognizing all caldesmon isoforms and followed by densitometry. All the experiments were made independently of one another with two HeLa 1469 populations deficient in caldesmon that were obtained as a result of two independent transductions. The data of two independent experiments were similar.

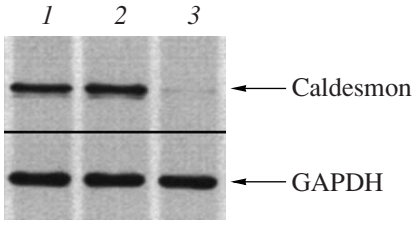

Fig. 1. The efficiency of suppression of caldesmon expression in HeLa 1469 cells by RNA-interference. The results of immunoblotting of lysates equal in total protein quantity are presented: lane *1*—lysate of non-transduced cells of human epithelial cell line HeLa 1469, lane *2* and *3*—lysate of control HeLa 1469 cells, expressing luciferase shRNA and expressing caldesmon shRNA correspondingly that were prepared 72 h after transduction. The upper range shows the membranes, stained with caldesmon-specific antibodies, the lower range shows the same membranes, stained with GAPDH-specific antibodies as a control of equal total protein quantity in different lanes.

Electrophoresis and immunoblotting. Cells were grown up to confluence. The medium was removed, cells were washed with phosphate buffer saline (PBS), containing 5.2 mM Na_2HPO_4, pH 7.4, 150 mM NaCl, then cells were lysed in twofold-concentrated sample buffer for electrophoresis (Laemmly method [28]) and were boiled for 5 min. Protein extracts were quantified using Shaffner-Weissmann method with amide black [29]. Proteins were resolved by 10–12% sodium lauryl sulfate-polyacrylamide gel electrophoresis (under 160 V voltage, 0.1% sodium laurylsulfate, Sigma, USA) by Laemmly method [28]. The quantity of applied samples was normalized using glyceraldehyde-phosphate-dehydrogenase (GAPDH) content in the samples. Immunoblotting was performed using 0.45 μm pore size PVDF membranes (Millipore, USA) as described [30]. For following caldesmon and GAPDH detection protein extracts were transferred in buffer, containing 0.025 M Tris, 0.192 M glycine, pH 8.3, 20% ethanol, 0.02% sodium laurylsulfate, pH 8.3 (transfer time 1 h, current strength 350 mA).

In this study we used primary earlier described rabbit polyclonal antibodies to caldesmon [31]. Primary mouse antibodies specific to GAPDH were kindly provided by D.V. Serebryanaya (the laboratory of Immunochemistry, Faculty of Biology, Moscow State University (MSU)). In this study we also used secondary antibodies specific to mice IgG and rabbit IgG, conjugated with horseradish peroxidase, and enhanced chemiluminescence detection kit for immunoblots (Amersham, USA). Quantitative analysis was performed using Image J software (Scion Inc., USA).

Cell stimulation and immunocytochemical staining of actin. Control Hela 1469 cells and cells with decreased caldesmon expression were cultured till 40–50% confluent monolayer formed at the cover-slip, then cells were deprived in the medium containing 0.1% FCS for 4 h and had been incubated in 30% FCS-con-

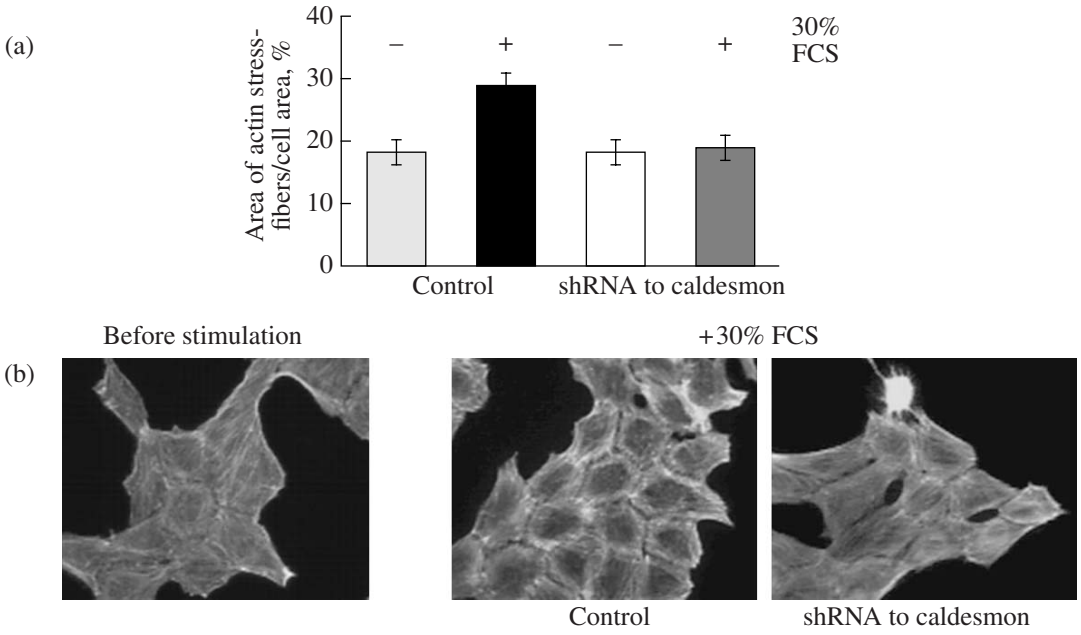

Fig. 2. Caldesmon influence on the actin stress-fiber formation in HeLa 1469 cells after their stimulation with serum. The quantity of polymeric actin was measured in control and caldesmon-deficient cells before and after their stimulation with falloidin, conjugated with fluorescent label as described in "Materials and Methods." The mean value (n = 30) ± standard deviation (a) and representative photographs of stained cells (b) are presented.

taining medium. The cells were washed once with phosphate buffer saline (37°C) and treated for 15 min with 3.7% paraformaldehyde, 0.2% glutaraldehyde, 0.1% Triton X-100 solution in buffer for fixation cytoskeleton proteins (CPFB), containing 10 mM MES, 150 mM NaCl, 5 mM EGTA, 5 mM sucrose, 5 mM $MgCl_2$, pH 6.1. Then cells were washed twice with CPFB buffer and incubated in CPFB buffer without sucrose, containing 0.5 mg/ml $NaBH_4$. Cells were washed with PBS and stained with TRITC-conjugated falloidin specific to actin in accordance to manufacturer's instructions (P1951, Sigma, USA). The fluores-

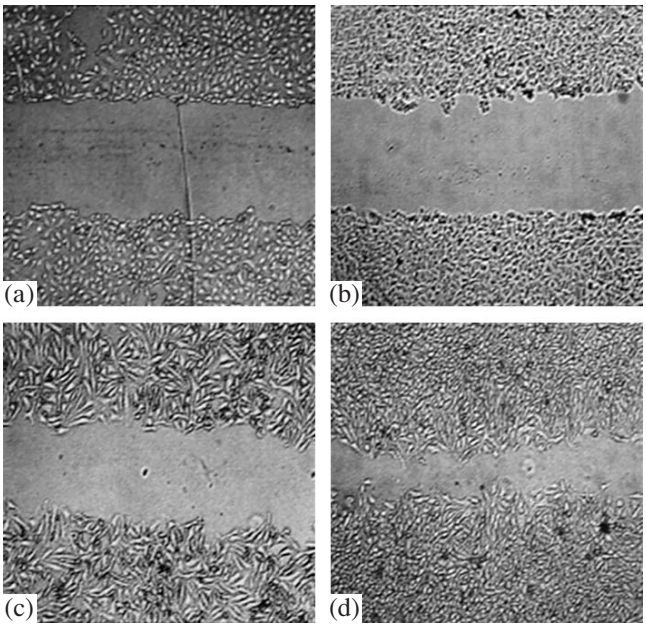

Fig. 3. Caldesmon influence on the rate of directed cell migration to the zone of monolayer injury. Phase-contrast pictures (10-fold enlargement) of monolayer of the control cells (a and c) and caldesmon-deficient cells (b and d) after the monolayer injury are presented: (a, b) immediately after the injury, (c, d) 12 h after the injury.

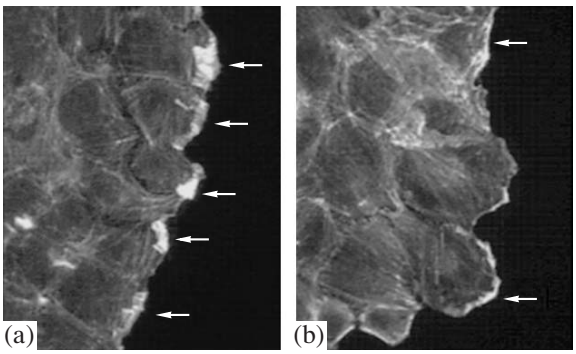

Fig. 4. Changes in actin cytoskeleton structure at the leading edge of migrating cells after the suppression of caldesmon expression. Polymeric actin staining with TRITC-conjugated falloidin in the control (a) and the caldesmon-deficient (b) cells, migrating from left to right to the scratch area are presented. The arrows show the arranged bundles of actin filaments, localized at right angle to the membrane at the leading edge of the control cells and non-arranged actin filament conglomerates at the leading edge of the caldesmon-deficient cells.

cent signal of TRITC-conjugated falloidin was registered with Zeiss Axiovert 200M microscope, equipped with CCD-camera AxioCam. Images were analyzed using Axiovision 3.1 software and Adobe Photoshop 6.0.

Quantitative image processing and quantitative analysis. The quantity of actin in filaments in the cells, pre- and post-stimulated with serum, and also in the cells actively migrating to a scratch zone were measured using MetaMorph Offline 7.1.7.0 software. The borders of the analyzed cell were selected and the mean minimal value of staining density in this region was assessed that was considered as base value below. The area of actin fibers that had the value of staining density more than twice the base level, was measured. The quantity of actin stress-fibers in cells was assessed as the ratio of stress-fiber area to the common area of the cell. Statistical analysis was carried out using Microsoft Excel 5.0 software.

Wound healing assay. Equal number of control or caldesmon-deficient cells HeLa 1469 was plated at plastic Petri dishes (or at cover-slips-depending on the experiment), so that 24 h after incubating cell culture took the form of 95% confluent monolayer. A scratch in cell monolayer was made by a sterile pipette tip (diameter 0.5 mm) and the culture medium was renewed. Cell migration to the scratch zone at the plastic dishes was observed and photographs of the same regions of the scratch were made every 15–30 min during 12 h after the cell monolayer injury. For this aim we used Zeiss Axiovert 200M microscope, equipped with CCD-camera AxioCam and box, in which general incubation conditions were provided (37°C and 5% CO_2). The degree of scratch "healing" was assessed as ratio of scratch square 12 h after the injury to that immediately after the injury.

When cover-slips were used, cells were incubated for 4 h after monolayer injury, then cells were fixed and probed with actin antibody as described above. The area occupied by actin filaments was measured in those cells that were located at the scratch edge and were first to fill up the injury zone.

RESULTS

Suppression of caldesmon expression in HeLa 1469 cells. HeLa 1469 cells express mainly low-molecular isoform of non-muscle caldesmon. Using the lentiviral transduction in these cells we obtained the cell population, where 99% cells stably expressed caldesmon shRNA and reporter protein GFP, which presence was detected with flow cytofluometry. In these cells endogenic caldesmon was significantly reduced comparing with non-transduced cells and with the cells, expressed the control shRNA (Fig. 1). 95% decrease of caldesmon expression comparing to the base level had occurred 72 h after transduction and stayed at low level during the time of experiments with that cell population (three weeks). We did not detect compensatory increase in expression of high-molecular smooth muscle caldesmon isoform.

Caldesmon takes part in stress-fiber formation during cell stimulation. We discovered, that decrease of intracellular caldesmon content did not lead to the remarkable changes in actin filament structure and localization in HeLa 1469 cells. The area occupied by stress-fibers in control and caldesmon-deficient cells did not differ and amounted to 18% of the total cell area (Fig. 2a). To test whether caldesmon is involved in the reorganization of actin cytoskeleton induced by chemoattractant we stimulated control and caldesmon-deficient cells with serum. In control cells serum caused remarkable accumulation of stress-fibers that tended to peripheric and circular, but not edge localization (Fig. 2b). Total quantity of fibers increased from 18 to 29% of cell area (Fig. 2a). However, such reorganization did not occur in cells with decreased level of caldesmon expression (Fig. 2b) and the area of fiber actin did not change in this cells. Thus, caldesmon appears to be involved inn actin stress fiber formation during cell stimulation.

Caldesmon inhibits cell migration. Using wound healing assay we tested whether caldesmon affects the rate of cell migration into empty area of injured monolayer. We found that the cells with lower caldesmon expression filled up injured area more efficiently (Fig. 3d) than the control cells (Fig. 3c). Analysis of 24 views in 2 independent experiments showed that suppression of caldesmon expression resulted in acceleration of cell migration to the injury more than by 25%. To exclude the possibility that it occurs because of increased proliferation of the caldesmon-deficient cells (which could have happened over 12 h experiment), we also analyzed the growth rate of the cells of either population and did not find any differences (data not shown).

Caldesmon organizes actin at the leading edge of migrating cells. Since in nonpolarized cells caldesmon binds cytoskeleton evenly in the whole cell, we studied its influence on actin in polarized cells that actively migrate to the zone of monolayer injury. Short bundles of actin filaments, localized perpendicular to the membrane, were found in the zone of protrusion activity of the control cells (Fig. 4a). There were almost no such structures in the caldesmon-deficient cells, and actin was localized in chaotic manner in the narrow zone near the leading edge membrane (Fig. 4b). These differences were observed only in polarized cells facing free area, but not in the cells of inner layers that did not have free space for migration. In addition, the total amount of polymeric actin was reduced in the external caldesmon-deficient cells, comparing to that of the control cells. The area occupied by actin filaments in these cells accounted for 13% of the whole cell area versus 19% in the control cells. Thus, in contrast to the nonpolarized cells caldesmon organizes actin mainly at the leading edge of the directly migrating polarized cells.

DISCUSSION

In the present study we show that caldesmon functions in cell as an actin organizer. It provides for actin assembly into large filamentous structures located differently depending on cell polarization. It is important that this effect is observed during cell stimulation and isn't seen in resting cells. The caldesmon-deficient cells migrated to the monolayer scratch faster than control cells, suggesting a role for caldesmon in regulation of actin dynamics at the leading edge of directly migrating cells.

In previous studies attempts have been made to reveal mechanisms of caldesmon activity in cells using overexpression or knocking down expression of this protein. However, there were no direct experiments studying caldesmon effect on the rate of cell migration. The available data suggest that caldesmon regulates assembly of large actin structures that play mechanical role in locomotion and contractile activity. In endothelium caldesmon was found important for the assembly of the stress-fiber ring, which contraction regulates endothelium permeability [17]. In smooth cells caldesmon plays a role in formation of dense actin core of podosomes—special structures involved in invasion [16, 26, 32]. Caldesmon participates in the reorganization of actin filaments in human trabecular meshwork cells [33]. Consistent with these observations, our data suggest that caldesmon assembles actin filaments and inhibits directed cell migration (Fig. 2).

Although the molecular mechanism of caldesmon function in cell requires further studies, it is likely to involve polymerization and bundling of actin filaments [13], alteration of actin filament nucleation and the filament branching [21], protection from fragmentation, for instance by gelsolin [19]. The joint result of all of these effects would be an increase in the length of actin filaments and number of their lateral aggregates, resulting in reduction of monomeric actin pool and short single fibers. The opposite changes are observed upon decrease in caldesmon intracellular content (Figs. 2b, 4).

The mechanism that couples changes in actin dynamics to cell motile activity is also unclear. The transient increase of caldesmon level in fibroblasts by caldesmon cDNA transfection reduced stress fiber contraction and weakened adhesion [15]. These data are consistent with inhibition by caldesmon of actomyosin ATPase and reciprocal relation between the stress-fiber tension and the strength of adhesions [34]. On the other hand, reduced adhesion strength is associated with increased rate of migration [2], and therefore the reduction of intracellular caldesmon content would lead to the reinforcement of adhesion strength and to the decrease of the migration rate. Since the migration rate wasn't measured for cells with increased caldesmon expression [15], the mechanism by which caldesmon affects the migration rate remains presently uncertain. Our finding that cell migration increases when caldesmon content is reduced does not conform the above mechanism. In addition, the rate of adhesion apparently is not changed in caldesmon deficient cells (Kudryashova T.V., unpublished observation). Altogether, these data argue against possibility that caldesmon affects the rate of migration through the regulation of the adhesion contact strength.

Our results suggest another mechanism of coupling the cytoskeletal effects of caldesmon to altered cell migration rate. That caldesmon promotes actin assembly indicates the reduction of the monomeric actin pool in the cell. We suggest that this slows down and/or inhibits actin turnover in the lamellopodium and reduces protrusion rates at the leading edge. It is noteworthy that marked actin assembly by caldesmon occurs in the lamellopodia of the actively migrating polarized cells. This does not happen when caldesmon expression is decreased (Fig. 4). In contrast, in nonpolarized cells caldesmon recruits actin into filaments evenly throughout the cell (Fig. 2). This indirectly suggests that caldesmon is redistributed in the cell in response to chemoattractant during directed migration. This is consistent with numerous data, consistent with intracellular caldesmon activity being under receptor control and regulated by with phosphorylation. Phosphorylation dynamics of caldesmon in the cell correlates with changes in motility activity [27, 35]. Thus, caldesmon phosphorylation appears to be the likely mechanism to transduce signal from the receptor, activated by chemoattractant, to the cell motile machinery.

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REFERENCES

1. A. J. Ridley, M. A. Schwartz, E. Burridge, et al., Science **302** (5651), 1704 (2003).
2. D. A. Lauffenburger and A. F. Horwitz, Cell **84** (3), 359 (1996).
3. J. A. Theriot and T. J. Mitchison, Nature **352** (6331), 126 (1991).
4. L. P. Cramer, T. J. Mitchison, and J. A. Theriot, Curr. Opin. Cell. Biol. **6** (1), 82 (1994).
5. J. V. Small and G. P. Resch, Curr. Opin. Cell. Biol. **17** (5), 517 (2005).
6. T. J. Mitchison and L. P. Cramer, Cell **84** (3), 371 (1996).
7. L. M. Machesky and K. L. Gould, Curr. Opin. Cell. Biol. **11** (1), 117 (1999).
8. A. Bershadsky, Trends Cell. Biol. **14** (11), 589 (2004).
9. T. D. Pollard and G. G. Borisy, Cell **112** (4), 453 (2003).

10. S. J. Winder and K. P. Ayscough, J. Cell. Sci. **118** (4), 651 (2005).
11. A. V. Vorotnikov, M. A. Krimskiy and V. P. Shirinskiy, Biochem. **67**, 1587 (2002).
12. M. J. Greenberg, C. L. Wang, W. Lehman, and J. R. Moore, Cell. Motil. Cytoskeleton **65** (2), 156 (2008).
13. R. Dabrowska, N. Kulikova, and M. Gagola, Protoplasma **224** (1-2), 1 (2004).
14. J. Kordowska, R. Huang, and C. L. A. Wang, J. Biomed. Sci. **13** (2), 159 (2006).
15. D. M. Helfman, E. T. Levy, C. Berthier, et al., Mol. Biol. Cell **10** (10), 3097 (1999).
16. R. Eves, B. A. Webb, S. Zhou, and A. S. Mak, J. Cell. Sci. **119** (9), 1691 (2006).
17. T. Mirzapoiazova, I. A. Kolosova, L. Romer, et al., J. Cell. Physiol. **203** (3), 520 (2005).
18. K. S. Warren, D. C. Shutt, J. P. McDermott, et al., Cell. Motil. Cytoskeleton. **34** (3), 215 (1996).
19. R. Ishikawa, S. Yamashiro, and F. Matsumura, J. Biol. Chem. **264** (13), 7490 (1989).
20. R. Ishikawa, S. Yamashiro, K. Kohama, and F. Matsumura, J. Biol. Chem. **273** (41), 26991 (1998).
21. Y. Yamakita, F. Oosawa, S. Yamashiro, and F. Matsumura, J. Biol. Chem. **278** (20), 17937 (2003).
22. V. B. Patchell, A. V. Vorotnikov, Y. Gao, et al., Biochim. Biophys. Acta **1596** (1), 121 (2002).
23. R. Huang, L. Li, H. Guo, and C. L. Wang, Biochemistry **42** (9), 2513 (2003).
24. E. A. Goncharova, V. P. Shirinsky, A. Y. Shevelev, et al., FEBS Lett. **497** (2-3), 113 (2001).
25. E. A. Goncharova, A. V. Vorotnikov, E. 0. Gracheva, et al., Biol. Chem. **383** (1), 115 (2002).
26. Z. Gu, J. Kordowska, G. L. Williams, et al., Exp. Cell. Res. **313** (5), 849 (2007).
27. R. D. Eppinga, Y. Li, J. L. Lin, et al., Cell. Motil. Cytoskeleton **63** (9), 543 (2006).
28. U. K. Laemmli, Nature **227** (5259), 680 (1970).
29. W. Schaffner and C. Weissmann, Anal. Biochem. **56** (2), 502 (1973).
30. H. Towbin, T. Staehelin, and J. Gordon, Proc. Natl. Acad. Sci. USA **76** (9), 4350 (1979).
31. K. G. Birukov, V. P. Shirinsky, A. V. Vorotnikov, and N. B. Gusev, FEBS Lett. **262** (2), 263 (1990).
32. T. Morita, T. Mayanagi, T. Yoshio, and K. Sobue, J. Biol. Chem. **282** (11), 8454 (2007).
33. B. T. Gabelt, Y. Hu, and J. L. Vittitow, Exp. Eye Res. **82** (6), 935 (2006).
34. A. D. Bershadsky, N. Q. Balaban, and B. Geiger, Annu. Rev. Cell Dev. Biol. **19**, 677 (2003).
35. J. Kordowska, T. Hetrick, L. P. Adam, and C. L. A. Wang, Exp. Cell Res. **312** (2), 95 (2006).