

Cofilin-like Protein Influences the Motility of *Amoeba proteus*

Wanda KŁOPOCKA¹, Katarzyna WIERZBICKA¹, Paweł POMORSKI¹, Patryk KRZEMIŃSKI¹ and Anna WASIK²

¹Department of Molecular and Cellular Neurobiology, ²Department of Cell Biology, Nencki Institute of Experimental Biology, Warszawa, Poland

Summary. The organization of *Amoeba proteus* cortical layer is highly associated with cofilin-like protein activity. This protein is involved in actin dynamics in the middle-anterior region of migrating cells, but does not take place in processes of the cortical network disorganization that occurred in the uroid. Cofilin homologue and actin co-localized at the leading edge, in the cortical and perinuclear cytoskeleton, in the area of cellular adhesion and in streaming endoplasm. Actin dynamics induced by cofilin-like protein are important for normal morphology and motility of *A. proteus*.

Key words: actin dynamic, *Amoeba proteus*, cofilin-like protein.

INTRODUCTION

Actin filaments in nonmuscle cells are highly dynamic and play an essential role in numerous cellular processes, including cell migration, endocytosis and cytokinesis. Organization of the contractile network in tissue cells as well as in amoebae is highly associated with activity of actin-binding proteins, including capping, severing, depolymerizing ones, as well as the motor proteins - myosins. Their function depends on the

activating signals, and it is regulated by different signal pathways.

The ADF/cofilins (ACs) are a family of small (15-20 kDa) proteins, expressed in all so far examined eukaryotic cells, that severs and depolymerizes ADP-filaments. ADF/cofilin was first discovered and purified in 1980 from embryonic chick brain extracts by Bamburg *et al.* (1980). Since then, the family was grown to include a number of related proteins, e. g. *Acanthamoeba* actophorin (Quirk *et al.* 1993) and *Dictyostelium discoideum* coactosin (de Hostas *et al.* 1993).

Phosphorylation is a principle regulator of ACs function. The activity of ACs is inhibited by phosphorylation at Ser-3 near the N terminus (Agnew *et al.* 1995) or Ser-2 in *Acanthamoeba castellanii* actophorin with the exception of related proteins from yeast and

Address for correspondence: Anna Wasik, Department of Cell Biology, Nencki Institute of Experimental Biology, Polish Academy of Sciences, Pasteur St. 3, 02-093 Warszawa, Poland; E-mail: a.wasik@nencki.gov.pl

Dictyostelium discoideum (Blanchoin *et al.* 2000). Phosphorylation blocks ACs interaction with ADP-actin filaments and monomers. LIM kinase (Yang *et al.* 1998, Arber *et al.* 1998) controls the activity of most ACs by phosphorylation. A specific phosphatase called slingshot activates ACs by removing the inhibitory phosphate (Niwa *et al.* 2002).

The main activity of ACs has been found from *in vitro* studies to be to increase actin filament turnover (Carlier *et al.* 1997, Maciver 1998, McGough *et al.* 2001). ACs accomplish this by depolymerizing filaments at their pointed ends, thereby providing a pool of actin monomers for a filament assembly, and severing actin filaments and consequently increase the number of filaments ends for polymerization (Bamburg *et al.* 1999, Carlier *et al.* 1999, Maciver and Hussey 2002). Together with profilin (and thymosin- β 4 in higher eukaryotes), ADF/cofilin (AC) and capping of barbed ends allow cells to maintain a high concentration of unpolymerized actin far from equilibrium (Pollard and Borisy 2003).

In the highly motile *A. proteus* the cortical cytoskeleton undergoes dynamic reorganization. During migration in the uroidal region contraction accompanied by solation leads to disorganization of the cortical system. Cytoskeletal proteins, including actin oligomers and monomers, are released into the endoplasmic streaming and flow to the top of advancing pseudopodia where, just beneath the plasma membrane, actin polymerization and cross-linking of microfilaments occur. Differences in the cortical system organization and aggregation are tightly related to the mechanism of the actin cytoskeleton assembly and contraction. RhoA- and Rac1-like proteins are involved in the regulation of these processes (Kłopocka and Rędowicz 2003, 2004; Kłopocka *et al.* 2005). Recently, we revealed that organization of the cortical layer in *A. proteus* is associated with the Arp2/3 complex activity (Pomorski *et al.* in press). In migrating amoeba actin polymerizes in Arp2/3 complex-dependent manner under the plasma membrane around the cell except distal parts: uroidal pole, retracting pseudopodia and tips of advancing ones. We also showed that the maximal content of filamentous actin in migrating amoeba is in the middle-posterior region, what was interpreted as an effect of the strong isometric contraction and aggregation of actin filaments in this region.

Here, we are focused on the localization and the effect of cofilin-like protein on *A. proteus*, including the role of endogenous cofilin in the morphology and motility of this amoeba *in vivo*.

MATERIALS AND METHODS

Cell culture. *Amoeba proteus* (strain Princeton) was cultured at room temperature in the standard Pringsheim medium. Amoebae were fed on *Tetrahymena pyriformis* twice a week, and used for experiments on the third day after feeding.

Immunoblotting. *A. proteus* cells were homogenised in a solution containing 10% sucrose, 20 mM imidazole (pH 7.0), 1 mM dithiothreitol, 50 mM NaCl, 10 mM sodium pyrophosphate, and 1 "Complete" tablet (Roche) per 25 ml of the solution.

Jurkat cells were centrifuged at 2500 g for 5 min, washed in PBS, and resuspended in lysis buffer containing: 1% Nonidet P-40, 120 mM NaCl, 50 mM Tris/HCl, pH 7.5, and freshly added proteinase inhibitors.

Immunoblotting procedure with the antisera against: α -actin (Sigma) and cofilin (Sigma) was carried out according to Kłopocka and Rędowicz (2004).

Immunolocalization studies. The distribution of cofilin-like protein was examined by indirect immunocytochemistry in migrating amoebae. Migrating cells were fixed and permeabilized according to Kłopocka and Rędowicz (2004). They were incubated overnight at 4°C with polyclonal antibody against human cofilin at a dilution of 1:20. This was followed by incubation with Alexa Fluor 488-conjugated anti-mouse secondary antibody (Invitrogen) at a dilution of 1:200 for 60 min at 37°C and then washing in PBS solution. For simultaneous assessment of F-actin distribution, cells were additionally stained by Alexa Fluor 546-phalloidin (Invitrogen). The stain was visualized with the confocal laser-scanning microscope (CLSM) Zeiss LSM 510. For co-localization measurements double laser and double excitation was used. No cross excitation or fluorescence bleed-through were observed (data not shown).

Locomotion studies. The effect of the inhibition of endogenous cofilin on *A. proteus* was examined by observation of living amoebae after microinjecting them with polyclonal antibody against human cofilin (final concentration 25 μ g/ml) diluted in Pringsheim medium. Controlled cells were microinjected with Pringsheim medium and the solution in which antibody was supplied (50% glycerol, 20 mM sodium phosphate pH 7.5, 150 mM NaCl, 1.5 mM NaN₃, 1 mg/ml BSA (data not shown).

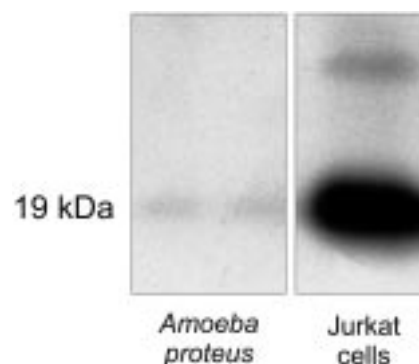


Fig. 1. Detection of cofilin-like protein. After separation of sodium dodecyl sulfate-polyacrylamide gel electrophoresis 25 μ g of *Amoeba proteus* homogenate proteins as well as 25 μ g of Jurkat cells lysate were probed with antibody against human cofilin, followed by detection with HRP-conjugated secondary antibody.

Microinjections were carried out according to Kłopocka and Rędownicz (2004).

RESULTS

Detection and localization of the cofilin-like protein

The cofilin-like protein from *Amoeba proteus* with apparent mass of about 19 kDa (Fig. 1), similar to the mass of cofilin from Jurkat cells, was identified by immunoblot analysis with specific polyclonal antibody raised against human cofilin. Jurkat cells express cofilin, so lysate of these cells was used as a positive control to confirm specificity of the signal detected in *A. proteus* homogenate.

Figure 2 presents CLSM optical sections through migrating amoebae which show the intracellular distribution of amoeba cofilin homologue. Figures 2A, B, C are optically sectioned images at 7.6 μm above the glass substrate. They show the cofilin-like protein (Fig. 2A), F-actin (Fig. 2B) distribution and their co-localization (Fig. 2C) on the ventral side of the migrating amoeba. Amoeba actin and cofilin are accumulated at the close contact site with substratum in the area where adhesive structures are formed. Optical sectioned images recorded at 15.2 μm from the glass substrate (Figs 2D, E, F) show the distribution of cofilin homologue (Fig. 2D), F-actin (Fig. 2E) and their co-localization (Fig. 2F) in the middle part of the cell sectioned along the longitudinal axis.

Cofilin-like protein localized in the middle-anterior region. It was concentrated in three different cell areas

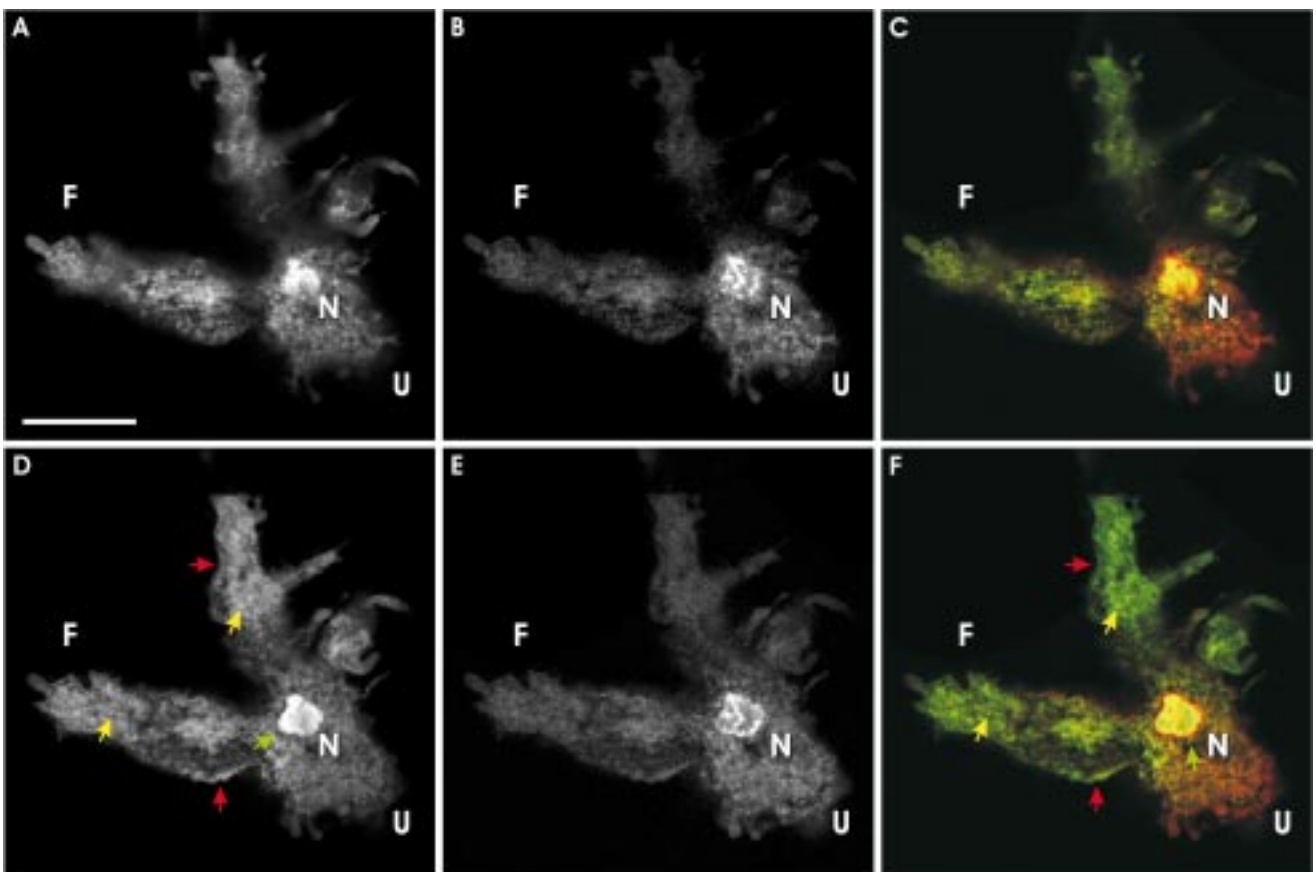


Fig. 2. Localization of cofilin homologue in migrating *Amoeba proteus*. Optically sectioned images of fluorescein and rhodamine staining at 7.6 μm (A-C) and 15.2 μm (D-F) from the glass substrate. Immunofluorescence using polyclonal antibody against human cofilin (A, D). Arrows point to areas where cofilin-like protein is recruited. Alexa Fluor 546-labelled phalloidin for F-actin (B, E). Co-localization of amoeba cofilin and F-actin (C, F). Red, green and yellow arrows show cortical layer, perinuclear area and adhesive region, respectively. F - front, N - nucleus, U - uroid. Scale bar: 50 μm .

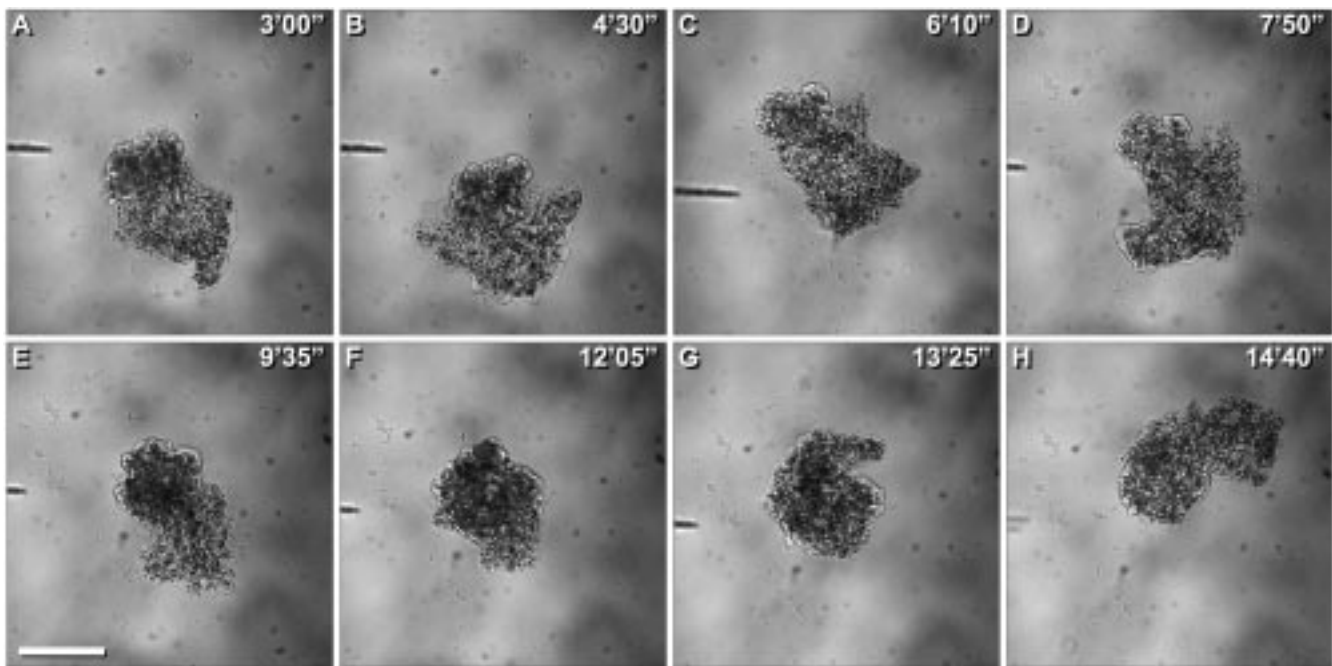


Fig. 3. Behavior of *Amoeba proteus* after microinjection of polyclonal antibody against human cofilin. Note the atypical mode of migration. Scale bar: 100 μm .

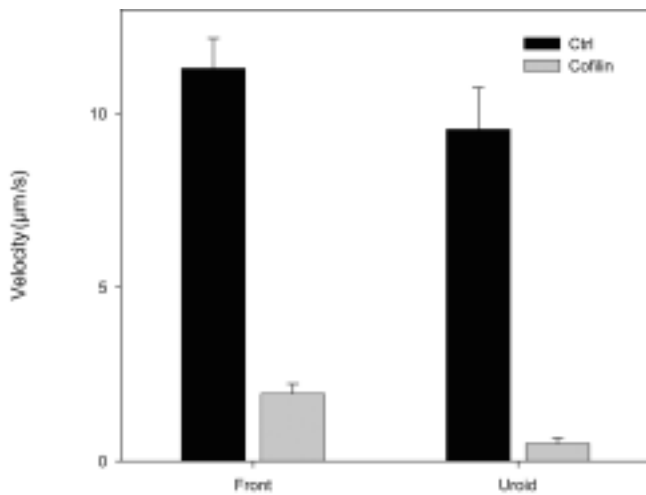


Fig. 4. The effect of blocking endogenous cofilin on the rate of frontal progression and uroidal retraction during migration of *Amoeba proteus*. Cofilin-like protein was blocked by microinjection of amoebae with polyclonal antibody against human cofilin.

and accompanied by F-actin: in the cortical network (Figs 2D and 2F, red arrows), in the perinuclear cytoskeleton (Figs 2D and 2F, green arrow) and in the area

where adhesive structures are developed (Figs 2D and 2F, yellow arrows).

Effect of inhibition of endogenous cofilin on locomotion of *A. proteus*

The effect of blocking the amoeba endogenous cofilin on the morphology and migration of amoebae was examined by microinjection of migrating cells with polyclonal antibody against human cofilin. Control cells were injected with the Pringsheim medium and with the solution in which antibody was supplied. They resumed their locomotion within 1 min. The mode and the velocity of migration were not changed (data not shown). Control experiments were consistent with data observed by Kłopocka and Rędownicz (2003, 2004).

Figure 3 demonstrates the results of blocking the endogenous cofilin-like protein by microinjecting migrating amoeba with the polyclonal anti-cofilin antibody. Observed, drastic changes in the morphology and rate of migration seem to be irreversible - each experiment was recorded for 60 min and the behaviour of amoebae was not changed. Amoebae started to migrate within 2-3 min after treatment. The cells were strongly contracted with inhibited formation of new fronts and disordered polar-

ization. They were also strongly attached to the substratum compared to control. A quantification of the effect of cofilin-like protein blocking on amoeba locomotion is presented in Fig. 4. Treatment of amoebae with antibody led to an inhibition of the frontal edge progression (above 90%) as well as the uroid retraction (about 80%), when compared with control amoebae.

DISCUSSION

Cell motility bases on actin assembly and disassembly, formation and disorganization of special cytoskeletal structures and contraction of the acto-myosin system. These processes are involved in creating and maintenance of cellular polarization, frontal protrusion, cell adhesion and tail region contraction connected with de-adhesion. Cell motility relies on the correct spatial and temporal reorganization of actin cytoskeleton that is regulated by numerous actin-binding proteins.

ADF/cofilins have been recognized as a family of essential, conserved, widespread acting-binding proteins important *in vivo* for the acceleration of actin filaments turnover during cell motility (Pantaloni *et al.* 2001) and cytokinesis (Gunsalus *et al.* 1995, Nagaoka *et al.* 1995).

Actin filament disassembly is necessary for polarized protrusion during cells migration. ACs localize to sites of intense actin filaments turnover (Bamburg and Bray 1987, Svitkina and Borisy 1999) including lamellipodium of crawling fibroblasts (Dawe *et al.* 2003), lamellipodium of keratocytes (Pollard and Borisy 2003), leading edge of *Acanthamoeba* (Quirk *et al.* 1993). A significant accumulation of cofilin was also observed at the leading edges of protruding pseudopodia in flattened cells of *D. discoideum* (Aizawa *et al.* 1995, 1997). In migrating tissue cells intense actin polymerization in the front of lamellipodia forms so-called dendritic brush (Svitkina and Borisy 1999), and is responsible for membrane displacement. Actin polymerization at the leading edge of migrating *A. proteus* seems to be rather insufficient in providing the driving force for a membrane displacement (Pomorski *et al.* in press). Microfilaments in fronts of advancing pseudopodia run mostly parallel to the plasma membrane (Wehland *et al.* 1979), and their formation is independent on the Arp2/3 complex. However, the reconstruction of the cortical layer in advancing fronts is necessary for maintenance of polarized migration. Actin filaments turnover in cortical cytoplasm, except tips of protruding pseudopodia is necessary for the formation of three dimensional contractile network

that activity generates force necessary for endoplasmic streaming, fronts protrusion, de-adhesion and retraction of the uroidal region.

Our results show that organization of the cortical layer in *A. proteus* is highly associated with cofilin-like protein activity. This protein is involved in actin dynamics in the middle-anterior region of migrating cells, but does not take place in processes of the cortical network disorganization occurring in the uroid. Observations on amoebae microinjected with the fluorescence G-actin (Gawlitta *et al.* 1980, Stockem *et al.* 1983) indicated a rather high rate of actin exchange between cytoplasmic matrix and the cell cortex in the middle-anterior region. Optical sectioning observation clearly indicated that cofilin homologue and actin co-localized at the leading edge, in the cortical and perinuclear cytoskeleton, in the area of cellular adhesion and in streaming endoplasm of this amoeba (Fig. 2). We suggest that cofilin-like protein is engaged in the regulation of actin filament turnover at regions of dramatically reorganizing actin cytoskeleton: at leading edge where the reconstruction of the cortical layer starts from elements of actin cytoskeleton bringing here by endoplasmic streaming (Grębecki 1982), in the cortical and perinuclear network where the processes of Arp2/3 complex-dependent actin polymerization occur, in the formation of adhesive structures, and in actin depolymerizing and severing in endoplasmic streaming.

In vivo experiments with inhibition of cofilin-like protein have revealed that this protein is a key player in the regulation of actin dynamics in *A. proteus*, and it is necessary for the proper cell migration. Cells lacking cofilin or over-expressing constitutively active LIM kinase have impaired locomotion (Arber *et al.* 1998, Chen *et al.* 2001), and those over-expressing cofilins are more motile (Aizawa *et al.* 1996).

Blocking endogenous cofilin by microinjecting amoebae with antibody against human cofilin caused the distinct and irreversible changes in the locomotive shape of the examined amoeba (Fig. 3) and significant (about 90%) inhibition of their migration (Fig. 4). Slowing down of actin filaments turnover leads to the strong contraction and the adhesion, inhibition of new fronts formation and polarization disorders. These changes seem to be related to disturbances in the reconstruction of the cortical layer at the leading edge, reorganization of the contractile network in the middle-anterior region and formation of adhesive structures.

It may be concluding that actin dynamics induced by cofilin-like protein are important for normal morphology and motility of *Amoeba proteus*.

Further studies are needed to clarify the function of cofilin-like protein during amoeba migration and the role of its activation and inactivation.

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