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Short Communication

Response of *Amoeba proteus* to Microinjection with Active Rac1 and RhoA Proteins

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Summary. Molecular mechanisms underlying the unique locomotion of highly motile free-living *Amoeba proteus*, a widely used model of amoeboid movement, still remain unknown. Recently, we have shown that blocking amoeba endogenous Rac- and Rho-like proteins led to the distinct and irreversible changes in the behaviour of these large locomoting cells and to a significant inhibition of their locomotion. To further elucidate the mechanism of Rho pathway, we tested the effect of introduction of active recombinant human RhoA and Rac1 proteins on amoebae cells. While the degree of the inhibition of migration of cells treated with both proteins seems to be similar to cells microinjected with anti-RhoA and anti-Rac1 antibodies, there are distinct differences in cell behaviour and morphology when compared with the blocked phenotypes. The results indicate the important and complex role of Rho-family proteins in amoebae migration.

Key words: *Amoeba proteus*, cell behaviour, cytoskeleton, locomotion, Rac, Rho.

INTRODUCTION

Rho family low-molecular GTP-binding proteins have been found in all eukaryotic organisms - from amoebae (Rędowicz and Korn 2000) and plants (Valster *et al*. 2000) to humans (Hall 1998). It has been known from more than a decade now that its members, Rho, Rac and Cdc42, govern many cellular functions associated with the actin-based cytoskeleton such as cell motility, endocytosis and exocytosis, muscle contraction, neurite outgrowth or cytokinesis (Takai *et al*. 2001). These proteins are active in GTP-bound form and inactive in GDP-bound form. Since their intrinsic GTPase activity is very low, the hydrolysis is enhanced by several regulatory proteins. GDP to GTP exchange is stimulated by guanine nucleotide exchange factor (GEF), GTPase-activating proteins (GAPs) increase the intrinsic rate of GTP hydrolysis and, in addition, guanine nucleotide dissociation inhibitors (GDIs) inhibit both the exchange of GTP and the hydrolysis of the bound GTP (Takai *et al*. 2001). It has been found that microinjection of active RhoA or its constitutively active mutant (Val14RhoA) into quiescent fibroblasts promotes stress fibers and focal adhesion assembly, active Rac1 stimulates

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lamellipodia and membrane ruffles formation, and activation of Cdc42 results in producing filopodia and associated adhesion complexes (Ridley 1995, Hall 1998). Rnd1, also a member of Rho family, promotes disassembly of actin filament structures and loss of cell adhesion (Nobes *et al*. 1998). Rho family proteins exert their functions through the activation of target proteins that upon binding to the active form of Rho family member are activated and affect their specific substrate proteins. RhoA binds, for example, to Rho-associated kinase (ROCK or ROK) that, in turn, phosphorylates and affects several cytoskeletal proteins such as myosin light chain phosphatase, myosin regulatory light chains, vimentin, cofilin or ezrin/moesin/radixin (Rędowicz 1999). Rac1 and Cdc42 activate, among others, PAK kinase (p21-activated kinase) that phosphorylates myosin regulatory light chains, myosin light chain kinase or amoeboid myosin I heavy chain (Brzeska *et al*. 1999, Daniels and Bokoch 1999).

The role of Rho family proteins in amoebae remains practically an unexplored area despite the distinct organization of actin-based cytoskeleton in amoeba cells (Stockem and Kłopocka 1988). Godbold and Mann (2000) have shown that expression in *Entamoeba histolytica* of C3 transferase, the specific RhoA inhibitor, resulted in the inhibition of its cytolytic activity thus indicating the involvement of Rho-dependent signal transduction in amoeba pathogenicity. Our previous observations that inhibition of endogenous Rac1-like and RhoA-like proteins leads to the significant and irreversible changes in morphology and locomotion of *Amoeba proteus* also confirm the crucial role of these small GTPases in protozoans, and suggest a possibility of a different mechanism of the Rho-mediated pathway (Kłopocka and Rędowicz 2003).

Here, by observing amoebae microinjected with active recombinant human Rac1 and RhoA, we attempted to further elucidate the role of Rho family proteins in *Amoeba proteus.*

MATERIALS AND METHODS

Cell culture

Amoeba proteus (strain Princeton) was cultured at room temperature in the standard Pringsheim medium [0.848 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.081 mM $MgSO_4 \cdot 7H_2O$, 0.112 mM $Na_2HPO_4 \cdot 2H_2O$, 0.349 mM KCl, 0.007 mM $FeSO_4 \cdot 7H_2O$; pH 6.8-7.2]. Amoebae were fed on *Tetrahymena pyriformis* twice a week and always used for experiments on the third day after feeding.

Protein samples

The recombinant human fusion GST-RhoA and GST-Rac1 proteins (GST - glutathione S-transferase) were expressed in the standard *E. coli* expression system using pGEX-2T vector as it has been described by Self and Hall (1995). The plazmids carrying the wild type RhoA and Rac1 proteins were from Dr. S. Gutkind laboratory (NIH, Bethesda). The expressed proteins were purified to homogeneity using glutathione-Sepharose 4B beads (Amersham, Pharmacia) and stored at -20°C, after an overnight dialysis at 4°C, in a buffer containing 25 mM Tris-HCl, 50 mM KCl, 4 mM EGTA, 20% glycerol, 0.2 mM DTT, 0.1 mM PMSF, 1μM GDP, and a set of protein inhibitors ("Complete" tablets from Roche). The exchange of GDP into GTP bound into the active site was essentially based on the method described by Yamamoto *et al*. (1990). Briefly, protein aliquots were taken directly before the experiment, adjusted to 12.5 mM Tris HCl, 5 mM EDTA and 0.5 mM DTT, supplemented with 2 mM GTP (from Sigma, USA) and incubated for 10 min at 30°C following the addition of $MgCl_2$ up to 10 mM final concentration. The GTP-bound RhoA and Rac1 were used for microinjection within 30 min after treatment.

Locomotion studies

The effect on *Amoeba proteus* was examined at room temperature by observation of living amoebae after microinjecting them with GTP-bound GST-Rac1 and GST-RhoA. The final concentrations of microinjected proteins after 1:10 dilution with Pringsheim medium were 45 μg/ml for GST-RhoA, and 45 and 110 μg/ml for GST-Rac1, which are within the range used for studies on fibroblasts (Ridley 1995). Control cells were microinjected with either Pringsheim medium or a buffer in which the proteins were prepared (see the Protein samples section). In each experiment, not less than seven amoebae were examined. Microinjections were carried out directly on standard microscopic slides with glass micropipettes held in an Eppendorf micromanipulator. After each microinjection the sequence of up to 500 DIC Nomarski images has been acquired. The time-lapse (5-s intervals) images have been registered using cooled CCD camera Retiga 1300 (QImaging Co., Canada) connected by firewire interface to PC computer running AQM advance 6-image acquisition software (Kinetic Imaging Ltd., U. K.). The same computer program was used to assess the velocity of locomotion of the frontal edge(s) and retraction of the uroid. The rate of migration of both uroidal and frontal parts were calculated separately.

RESULTS AND DISCUSSION

Cells microinjected either with the control buffers (Figs 1a and 1b) or with the GTP-bound forms of Rac1 (Fig. 2a) and RhoA (Fig. 2b) were observed and recorded for about 30 min after treatment, and the results are demonstrated by selected images.

Amoebae microinjected with Pringsheim medium (Fig. 1a) or the protein buffer (Fig. 1b) resumed the normal migration within two to five minutes after the treatment. Amoebae microinjected with GTP-binding

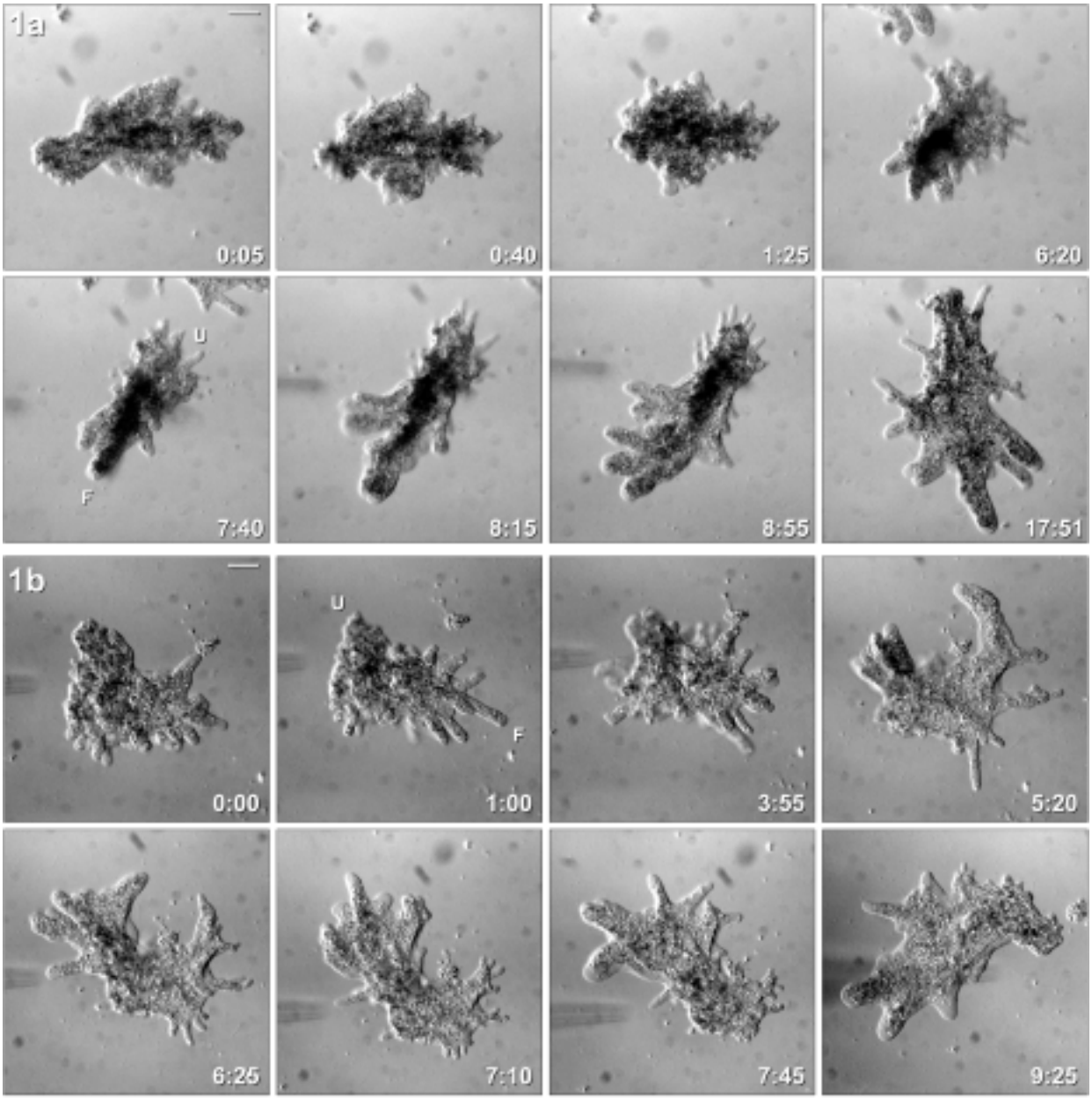

Fig. 1. Behaviour of *Amoeba proteus* after microinjection with either Pringsheim medium (a) or the buffer in which proteins were prepared (b). Numbers in the left bottom corner of each panel in this figure and in Fig. 2 reflect time (in min) after the treatment. Scale bar - 50 µm.

proteins remained irreversibly changed during the course of the experiment (Fig. 2a and b).

Cells introduced with 45 µg/ml Rac-GTP (Fig. 2a) attempted to migrate within three to four minutes after injection but their locomotive shape as well as the rate of migration were changed in comparison to control cells. They also did not appear to be so flat as the cells treated with anti-Rac antibodies (Kłopocka and Rędowicz 2003), they extended few wide pseudopodia that were able to advance in various directions. Adding about three times more of protein (110 µg/ml) caused even more pronounced changes (not shown).

Cells treated with Rho-GTP (Fig. 2b), contrary to cells treated with anti-RhoA antibodies or C3 trans-

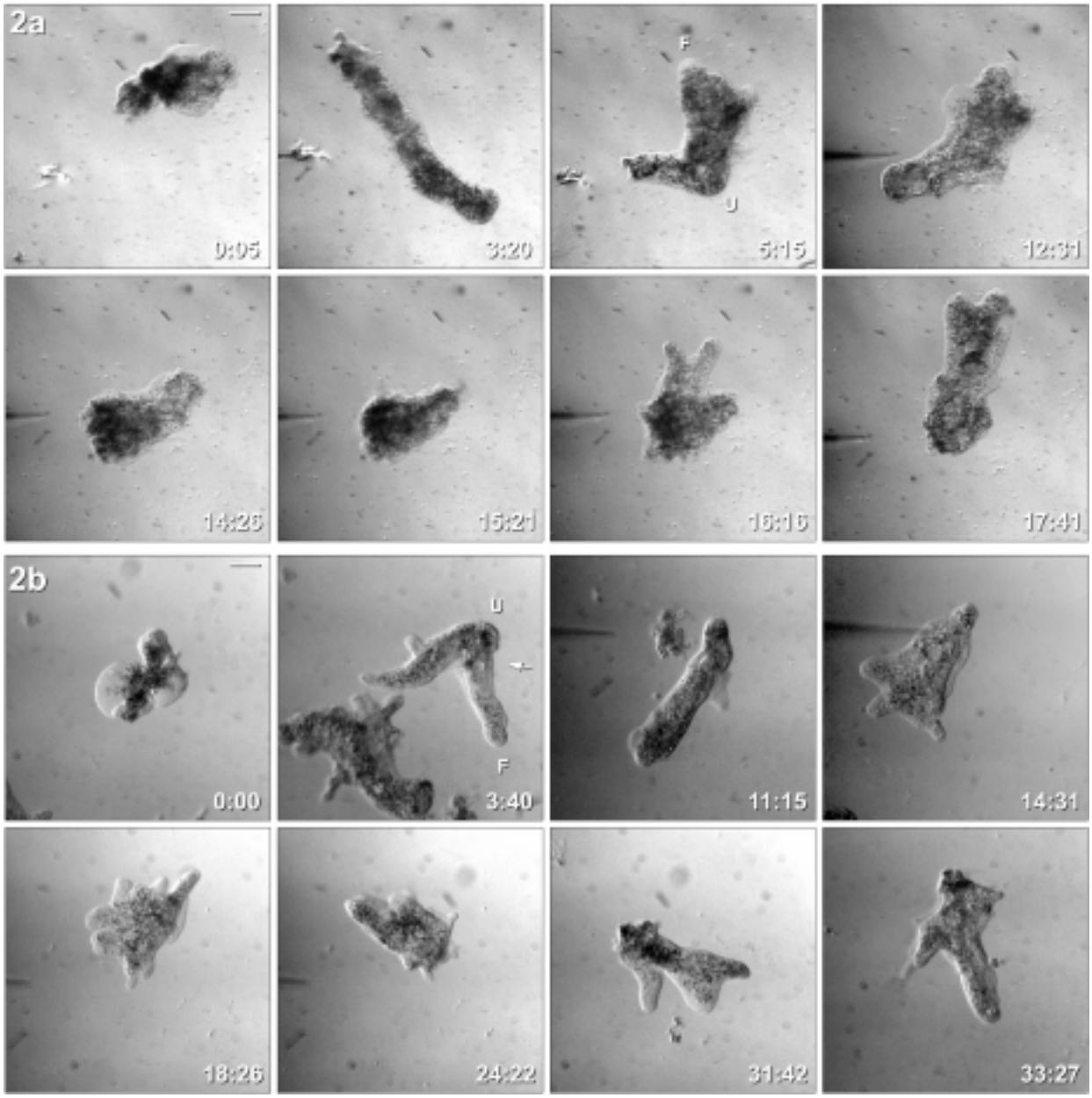

Fig. 2. Behaviour of *Amoeba proteus* after microinjection with the GTP-bound GST-Rac1 (a) and GTP-GST-RhoA (b) proteins at the 45 µg/ml concentration. Scale bar - 50 µm.

ferase (Kłopocka and Rędowicz 2003) were able to dislocate, did not round up and formed up to few, sometimes very long, protrusions.

Quantification of the migration rates of the cells microinjected either with control buffers or with active RhoA and Rac1 proteins, calculated separately for front and uroid, is presented in Fig. 3. Advancing of the frontal edges was inhibited about 41% and 48% when cells were treated with 45 µg/ml of RhoA and Rac1, respectively. The rate of the uroidal retraction was also lower than that of control cells, with the observed inhibition of about 50% for RhoA and 45% for Rac1 (Fig. 3a).

It is noteworthy that amoebae microinjected with Rac or Rho exhibited a kind of sequential pattern of migration in which, after several minutes of impaired locomotion, there were several periods lasting up to ten seconds

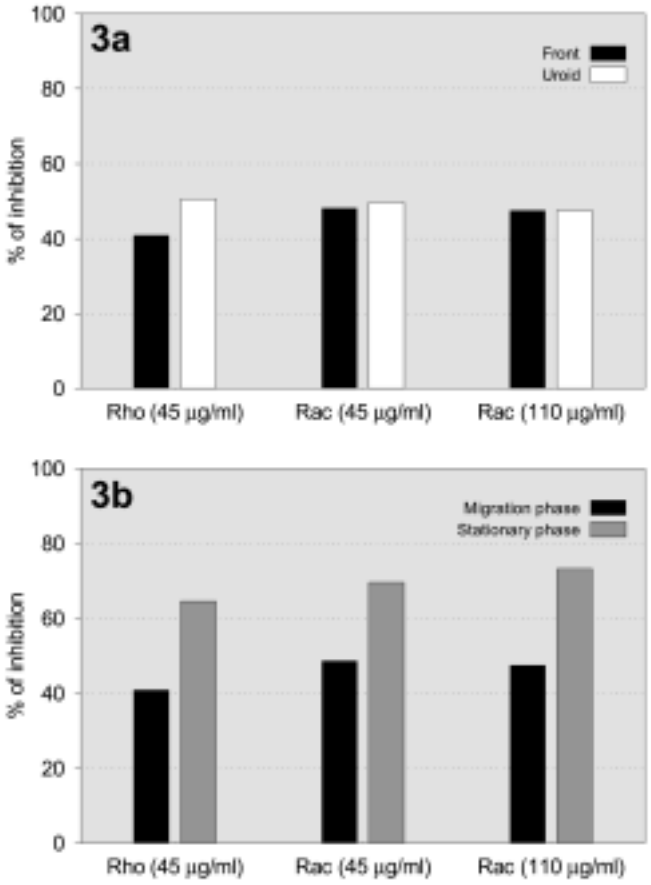

Fig. 3. Effect of introducing of active RhoA (45 µg/ml) and Rac1 (45 µg/ml and 110 µg/ml) on the rate of frontal progression and uroidal locomotion during the migration phase (a) and front advancing during the stationary phase (b). Results are expressed as % of the control cells migration rate.

when cells contracted, rounded up, strongly adhered to the glass surface and practically stopped locomotion (Figs 2a, 15:21; 2b, 24:22). Afterwards they resumed the migration and after several minutes they stopped again. During these periods (termed here as stationary phases) amoebae were yet able to extend protrusions but the rate of pseudopodial migration has significantly decreased when compared to cells treated with the buffer alone or during their migration phase. The inhibition, compared to control cells, was about 65% for cells treated with RhoA, about 70% for cells microinjected with 45 µg/ml of Rac1, and about 73% for cells treated with 110 µg/ml of Rac1 (Fig. 3b). Phenotypes observed during stationary phases resembled the phenotypes of amoebae in which endogenous Rac- and Rho-like proteins had been blocked with anti-RhoA and anti-Rac1 antibodies (Kłopocka and Rędowicz 2003). Interestingly, shortly after the resumption of the migration the inhibition of formation of the protrusion reached the overall frontal migration of treated amoebae (about 41% for RhoA and about 48% for both Rac1 concentrations). It is plausible to think that the stationary phase reflects the state in which GTP bound in the active sites of the introduced proteins has been *in vivo* exchanged to GDP (Takai *et al*. 2001).

It has been earlier observed that constitutively active Rac1 protein (Val12Rac) did not affect fibroblasts migration in the wound closure test (Nobes and Hall 1999). However, introducing constitutively active Rho A protein (Val14Rho) caused about 95% inhibition of wound closure (Nobes and Hall 1999). Constitutively active Cdc42 protein (Val12Cdc42), the member of Rho family that has not been found in different amoebae (Lohia and Samuelson 1996, Rędowicz and Korn 2000, Kłopocka and Rędowicz 2003) and in slime mold *Dictyostelium discoideum* (Wilkins and Insall 2001), had practically no effect on fibroblasts migration (Nobes and Hall 1999). The authors explain these results in terms of the cooperation and coordination in time and space of distinct pathways controlled by these Rho family proteins.

Our results, different from the ones reported for fibroblasts, reflect the distinct and complex nature of the Rho-family pathways in *Amoeba proteus*. Because there is very little known about amoebae Rho-family proteins, their structure and functions, it is impossible to speculate on the possible mechanisms underlying the observed phenotypes. Certainly, one should keep in mind the gross differences in the complexity and dynamics of amoebae actin-based cytoskeleton, and the fact that these cells can migrate even about 500 faster that the fibroblasts in the wound closure test. These free-living cells are always ready to act and they probably do not need for their survival the signal transduction machinery as it has evolved in metazoan cells. It is also quite possible that amoebae have more, unknown yet, members of Rho family as it has been found in *E. histolytica* (Lohia and Samuelson 1993, 1996) and *D. discoideum* (Wilkins and Insall 2001). Introducing the excess of one of them may shift their delicate balance and activate the other protein(s) pathway(s) that under normal conditions are not functioning during migration. The role of the amoebae Rho-family proteins certainly needs to be further investigated since, besides the contribution to the general knowledge, the results might have more practical aspect, as it has been shown by Godbold and Mann (2000) inactivation of Rho-dependent signal transduction

pathway in *Entamoeba histolytica* leads to decrease of its invasiveness.

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