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Nucleus-associated actin in *Amoeba proteus*

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Abstract

The presence, spatial distribution and forms of intranuclear and nucleus-associated cytoplasmic actin were studied in *Amoeba proteus* with immunocytochemical approaches. Labeling with different anti-actin antibodies and staining with TRITC-phalloidin and fluorescent deoxyribonuclease I were used. We showed that actin is abundant within the nucleus as well as in the cytoplasm of *A. proteus* cells. According to DNase I experiments, the predominant form of intranuclear actin is G-actin which is associated with chromatin strands. Besides, unpolymerized actin was shown to participate in organization of a prominent actin layer adjacent to the outer surface of nuclear envelope. No significant amount of F-actin was found in the nucleus. At the same time, the amoeba nucleus is enclosed in a basket-like structure formed by circumnuclear actin filaments and bundles connected with global cytoplasmic actin cytoskeleton. A supposed architectural function of actin filaments was studied by treatment with actin-depolymerizing agent latrunculin A. It disassembled the circumnuclear actin system, but did not affect the intranuclear chromatin structure. The results obtained for amoeba cells support the modern concept that actin is involved in fundamental nuclear processes that have evolved in the cells of multicellular organisms.

Keywords: *Amoeba proteus*; Immunocytochemistry; Nuclear actin; Nucleus

Introduction

Amoebozoa is a large and diverse group of protists with evolutionarily specific features. The actin cytoskeleton constitutes a basis for organization of their cells, especially of cellular processes (Christiani et al. 1986; Jeon 1973; Kłopocka et al. 1988; Stockem and Kłopocka 1988; Stockem et al. 1982). The phenomenon of amoeboid locomotion has been the subject of many studies, but has remained controversial for a long time. Despite the huge amount of data about the cytoplasmic actin cytoskeleton, the presence of intranuclear actin in amoebae cells remains poorly understood.

The large free-living amoeba, *Amoeba proteus*, has served as a classic object in various investigations for more than two hundred years. In spite of many reports and reviews (Goodkov et al. 2014; Jeon 1973, 1995; Yudin 1990), some biological aspects of this unique organism remain poorly studied. An intact nucleus of *Amoeba* reaches approximately 40 μm in diameter and its regular discoid shape (Page 1986) makes it an appropriate object for study.

The presence of actin in the cell nucleus is generally accepted at the present time (Falahzadeh et al. 2015; Gall 2006; Hofmann 2009; Wesolowska and Lénárt 2015). Nuclear actin is thought to have a broad range of functions. Specifically, it participates in maintaining the structure of the nucleus and in chromatin remodeling, transition and modification. Nuclear actin is also involved in such processes as

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transcription and nuclear export (Chen and Shen 2007; Gall 2006; Gieni and Hendzel 2009; Hofmann 2009; Maslova and Krasikova 2012; Miralles and Visa 2006; Visa and Percipalle 2010).

Nuclear actin has been demonstrated in some unicellular eukaryotes that belong to several phylogenetically distant groups. *Acanthamoeba castellanii* (Kumar et al. 1984), some ciliates (Benken and Sabaneyeva 2011; Katsumaru and Fukui 1982; Sehring et al. 2010), dinoflagellates (Soyer-Gobillard et al. 1996), and cellular and plasmodial slime moulds (Fukui 1978; Fukui and Katsumaru 1979; Jockusch et al. 1971, 1974) have been reported to possess actin in their nuclei.

Goldstein et al. (1977) were the first to detect a 42-kDa protein in the nucleus of *A. proteus* and to suggest that this protein could represent actin. The assumption that nuclear actin is mostly polymerized and involved in chromatin condensation was made in a subsequent paper (Rubin et al. 1978). Presumptive actin filaments have been found in amoeba nucleoplasm in several ultrastructural studies (Fukui and Katsumaru 1979; Gromov 1985; Lesson and Bhatnagar 1975). The presence of filamentous actin (F-actin) in contact with the cytoplasmic side of the *A. proteus* nuclear envelope was shown with the use of FITC-phalloidin staining (Grębecka et al. 1999). In addition, the existence of a special thick layer of actin filaments adjacent to the outer nuclear membrane and connected with cytoplasmic cytoskeleton was demonstrated (Pomorski and Grębecka 1993, 1995; Pomorski et al. 2000; Wasik et al. 2000).

However, the spatial organization and predominant forms of intranuclear actin in *A. proteus* still remain unknown. In the present study we have elucidated these issues by fluorescent labeling and immunocytochemical approaches.

Material and Methods

Culture conditions

Amoeba proteus (strain B) from the Amoebae Cultures Collection of the Institute of Cytology RAS (Goodkov et al. 2014, 2015) was used. Amoebae were cultivated at room temperature in the modified mineral Prescott solution (Prescott and Carrier 1964) and fed on the ciliate *Tetrahymena pyriformis* (strain GL) every 48 h according to the standard procedure (Kalinina and Page 1992; Podlipaeva et al. 2013). The *A. proteus* cells used in this study were at different stages of the cell cycle.

Antibodies

The following primary antibodies were used: polyclonal rabbit antibody against the N-terminal domain of the actin molecule (A2103, Sigma, USA) diluted 1:100, polyclonal rabbit antibody against the C-terminal domain (A2066, Sigma, USA) diluted 1:200, and a mouse monoclonal antibody that binds an epitope in a highly conserved region of actin (clone C4, MAB1501R, Millipore, USA) diluted 1:100.

Sequence analysis

The sequences were obtained from the GenBank database. The amino acid sequence of actin from *A. proteus* (NCBI: AAQ55807.1) and of α-actin from skeletal muscles (NCBI: NP_001091.1) were aligned to each other using NCBI Blast GenBank program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and BioEdit 7.0.9.0 editor.

Immunoblotting

Western immunoblotting was performed according to the procedure described earlier (Podlipaeva 2001; Goodkov et al. 2010). The markers ColorBurst (Sigma–Aldrich, USA) and Prestained Protein Ladder (Fermentas, Lithuania) were used as molecular weights markers. Purified rabbit skeletal actin was used as a control. After SDS-electrophoresis, proteins were transferred to a nitrocellulose membrane (Hybond) with subsequent treatment with a monoclonal (MAB 1501R, Millipore, USA) and polyclonal antibodies (A2103, A2066, Sigma, USA) against actin. An enzymatic color reaction was performed on membranes treated with monoclonal primary antibody. Zones of binding with anti-actin antibody were stained with secondary biotinyled antibody and Extravidin conjugated with alkaline phosphatase (Sigma–Aldrich, USA). The BCIP/NBT system (Santa Cruz Biotechnologies, USA) was used for localization of alkaline phosphatase labeled probes. A chemiluminescent reaction was performed on membranes treated with polyclonal primary antibody. Secondary antibody conjugated with horseradish peroxidase and fluorescent dyes ECL Select Western Blotting detection, reagent RPN 2235 (GE Healthcare, Ammersham, UK–Italy) were used to identify the protein bands. Blots were analyzed on the Chemidoc XRS using Quantity 1 soft.

Antibody labeling

The samples were prepared according to Hulsebos et al. (1984) using gelatinized microscopic slides and coverslips siliconized in Sigmacote SL2 solution (Sigma, USA). After squashing, *A. proteus* cells were fixed in 2% formaldehyde prepared in 96% ethanol at room temperature for 30 min. The preparations were then washed in 70% ethanol, PBS and permeabilized by 0.5% Triton X-100 for 10 min. After washing in PBS, the cells were treated with 10% bovine fetal serum in PBS for 10 min to prevent non-specific antibody binding. The incubation in primary antibody solution was carried out in a moist chamber at 4 °C overnight. After rinsing in PBS, the cells were incubated with FITC-conjugated goat anti-rabbit or anti-mouse secondary antibodies (Sigma, USA), dilution 1:200, in a moist chamber at room temperature for 1.5 h. After rinsing in PBS, preparations were mounted in Vectashield (Vector Laboratories, Inc., USA) containing 1 μg/ml DAPI

(AppliChem, Germany). In control preparations, incubation in primary antibodies was omitted.

Phalloidin treatment

Squash preparations were used for staining. After fixation and permeabilization, washed cells were incubated in 35 μg/ml TRITC-phalloidin solution in a moist chamber at room temperature for 30 min. The preparations were mounted in Vectashield with 1 μg/ml DAPI.

Latrunculin A treatment

A. proteus cells were incubated in latrunculin A-containing Prescott solution at room temperature for 2 h. Stock latrunculin A (Sigma) solution was diluted to a final concentration 1.5 μg/ml. After incubation, the cells were fixed and treated with TRITC-phalloidin as described above.

DNase I treatment

Squashed preparations of fixed and permeabilized cells were incubated in 9 μg/ml solution of fluorescent deoxyribonuclease I (Molecular Probes, USA) at room temperature for 30 min. Simultaneous staining with TRITC-phalloidin was also performed (35 μg/ml). The preparations were mounted in Vectashield with 1 μg/ml DAPI.

Confocal laser scanning microscopy and image processing

Preparations were examined by confocal laser scanning microscopy using the Leica TCS SP5 or Leica TCS SP8 (Leica-Microsystems). Argon 488-nm, helium-neon 543-nm and diode 405-nm lasers were used for fluorochrome excitation. Image section views and three-dimensional reconstructions were obtained by applying appropriate options in LAS AF Software (Leica-Microsystems). Digital images were cropped and measured using LAS AF and ImageJ. Relative contrast and brightness of images were adjusted with Adobe Photoshop CS5.

Results

Sequence analysis and immunoblotting

To determine whether the binding of antibodies used in this study resulted from an antigen-driven immune response, we compared amino acid sequences of actin from *A. proteus* (Fahrni et al., 2003) and of highly-conserved α-actin from human skeletal muscles. The sequences are available from GenBank (GB numbers AAQ55807.1 and NP_001091.1, respectively). Comparison of these sequences of length 374 amino acids (AAQ55807.1) and 377 amino

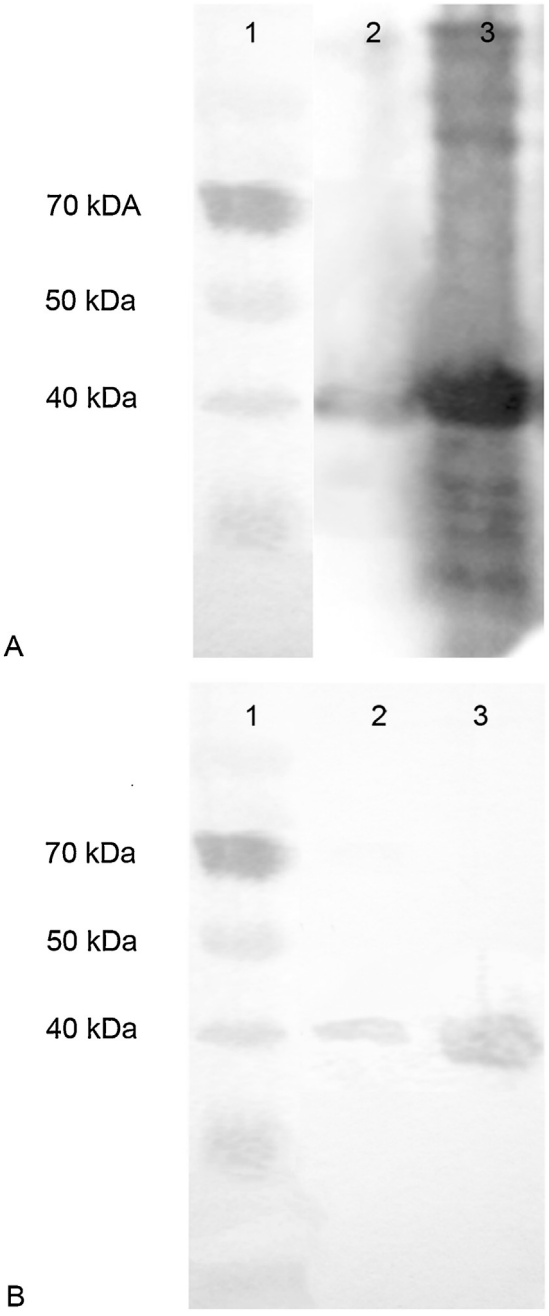

Fig. 1. Western blots after 10% SDS-electrophoresis, treated with polyclonal anti-actin antibodies followed by staining with anti-rabbit antibodies conjugated with horseradish peroxidase, ECL (A), and monoclonal anti-actin antibodies followed by staining with secondary anti-mouse biotinyled antibodies and Extravidin conjugated with alkaline phosphatase (B). In A and B, *lane* 1 – molecular markers Prestained Protein Ladder; *lane* 2 – *Amoeba proteus*, strain B, 5 μg of protein at the start gel; *lane* 3 – rabbit skeletal actin, 3 μg of protein at the start gel. *Note*: image taken from untreated blot after blotting and then combined with photo of the remaining lanes of the same blot after ECL reaction produced by Chemidoc-device.

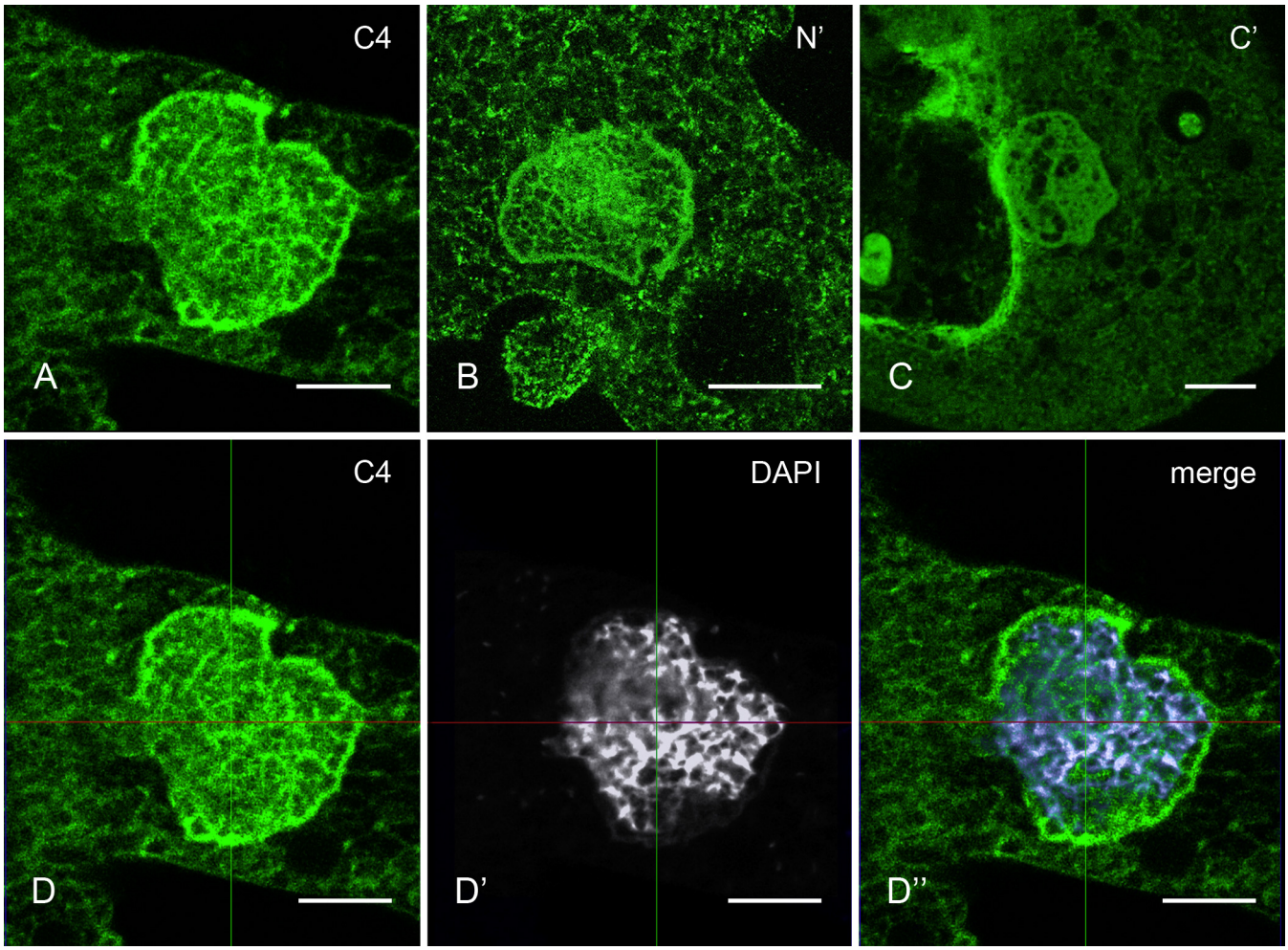

Fig. 2. Immunofluorescent staining of *Amoeba proteus* nuclei with antibodies against different regions of actin molecule. Staining with monoclonal anti-actin antibody, clone C4 (A), polyclonal antibody against the N-terminal (B) and the C-terminal domain of actin (C). (D) Distribution of actin in the nucleus of *Amoeba proteus* and co-localization with chromatin strands (C4 anti-actin antibody labeling). Scale bars: 10 μm (A, D–D"), 20 μm (B, C).

acids (NP_001091.1) showed 90% identity with 99% query cover (NCBI Blast). Pairwise alignment using the BioEdit 7.0.9.0 editor also demonstrated 88.6% identity (compared fragment of 377 amino acids). The results of the alignment are presented in Supplementary Table 1. The epitopes recognized by the antibodies appear to be located on the N- and C-terminal regions of the molecule – approximately 50–70 amino acid residues for MAB1501, clone C4 antibody; the first nine amino acid residues of the N-terminal region for A2103 antibody; and the C-terminal SGPSIVHRKCF sequence for A2066 antibody – according to the provider's data (Supplementary Table 1).

The specificity of antibody binding was then tested by Western immunoblotting. Binding with a polyclonal antibody occurred near the 40 kDa molecular marker (Fig. 1A, *lane* 1) and coincided with the samples from amoebae (Fig. 1A, *lane* 2) and rabbit skeletal actin (Fig. 1A, *lane* 3). On Fig. 1B staining is pale, but specifically stained bands are located in the same position (Fig. 1B, *lanes* 2 and 3). Thus, we suggested that antibodies used in the study are suitable for detecting actin in *A. proteus* cells.

Immunofluorescent microscopy

The presence of actin in the nucleoplasm of *A. proteus* and in the adjacent region of the cytoplasm was examined by immunostaining/labeling with a set of antibodies against different regions of actin molecule (see Section "Methods"). Immunofluorescent/confocal microscopy revealed staining of amoeba nuclei regardless of the antibody used in the study (Fig. 2). The results obviously suggest that the nucleus contains a pool of actin. Besides, a cytoplasmic layer adjacent to the nucleus was brightly stained with anti-actin antibodies (Fig. 2). The intensity of nuclear staining was comparable with that of the cytoplasm.

The analysis of confocal optical sections of the *A. proteus* nucleus showed the presence of an actin meshwork in the nucleoplasm (Fig. 2A–D). Examination of merged

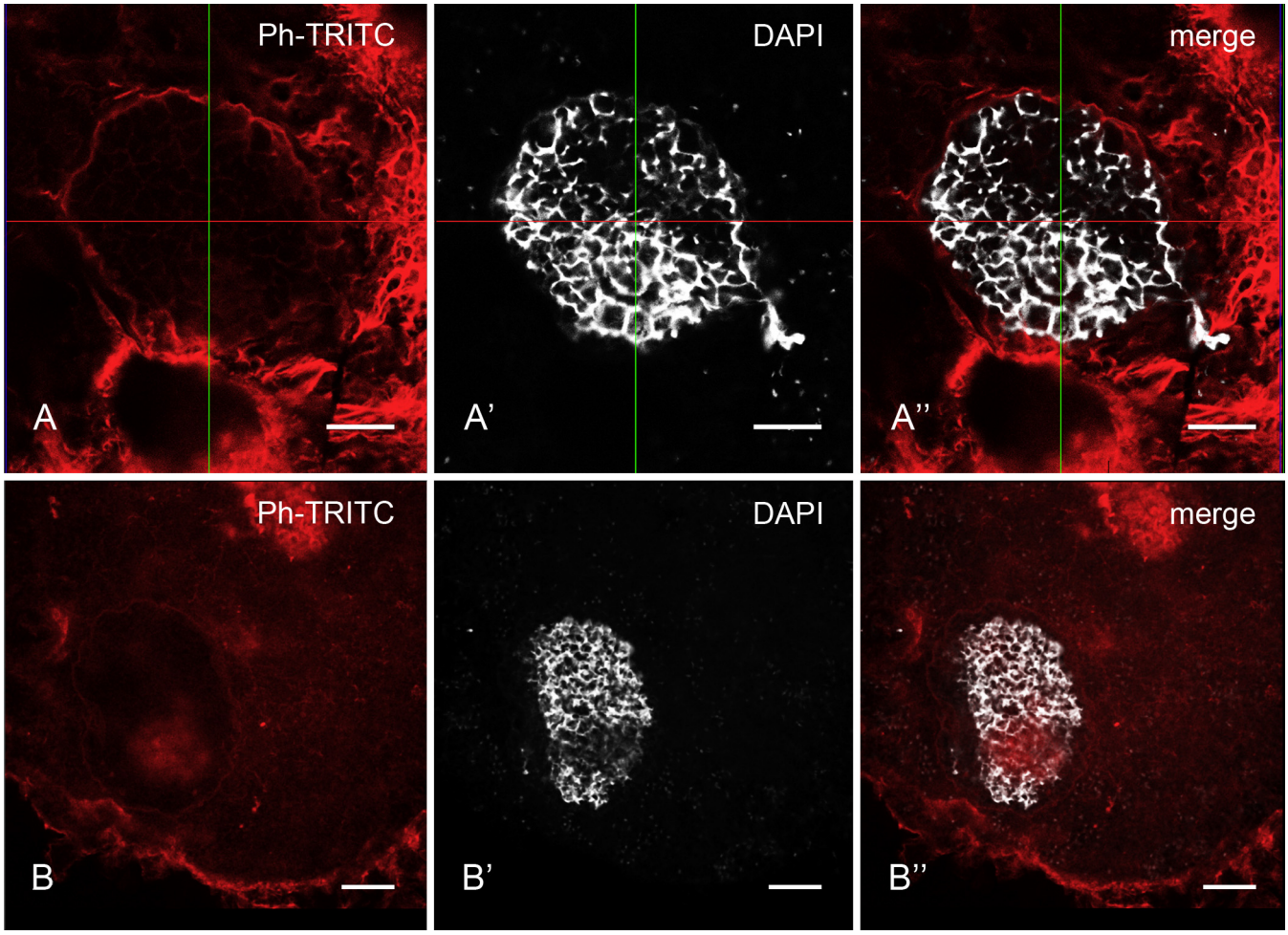

Fig. 3. Distribution of F-actin in the nucleus and adjacent cytoplasm of *Amoeba proteus* detected by staining with TRITC-phalloidin. Single confocal sections through the untreated cell (A–A") and treated with latrunculin A (B–B"). Scale bars: 10 μm.

images obtained after antibody and DAPI staining demonstrated almost complete co-localization of intranuclear actin structures and chromatin strands constituting an irregular meshwork (Fig. 2D–D"). Staining of the nucleus and the adjacent cytoplasmic regions indicated that actin forms a dense continuous layer between the two regions (Fig. 2A–D, D"). According to the analysis of the three-dimensional reconstruction of the nucleus this layer covered almost the entire surface of the nucleus (Fig. 4A).

TRITC-phalloidin staining

Inasmuch as a significant amount of actin was revealed with antibodies in *A. proteus* cells, including their nuclei, we then used the TRITC-phalloidin staining to determine whether the pool of actin could be in the polymerized form (F-actin). The cells were counterstained with DAPI for chromatin visualization. Examination of confocal sections demonstrated that the signal in the nucleoplasm was practically absent (Fig. 3A–A"). Instead the nuclei were surrounded by numerous actin filaments, whose bundles were in close association with the outer surface of the nuclear envelope (Figs 3A–A", 4B). Moreover, these filaments and bundles were associated with a loose network of cytoplasmic microfilaments (Fig. 4B). The intensity of TRITC-phalloidin staining was similar in the area adjacent to the nucleus and in the rest of the cytoplasm.

Latrunculin A treatment

The supposed architectural function of actin filaments in *A. proteus* cells was tested by treatment with the actin-depolymerizing agent latrunculin A, followed by staining with TRITC-phalloidin and DAPI. After 2 h of incubation with latrunculin A, the cells of *A. proteus* lost their motility and became rounded. Distinct filaments and bundles were not observed any more, and the intensity of phalloidin labeling in the entire cytoplasm was relatively lower than in untreated cells. A well-stained actin layer on the outer surface of the nucleus was no longer observed (Fig. 3B–B"). Nevertheless, the nuclear profile was still distinguishable (Fig. 3B'). Moreover, counterstaining with DAPI demonstrated a typi-

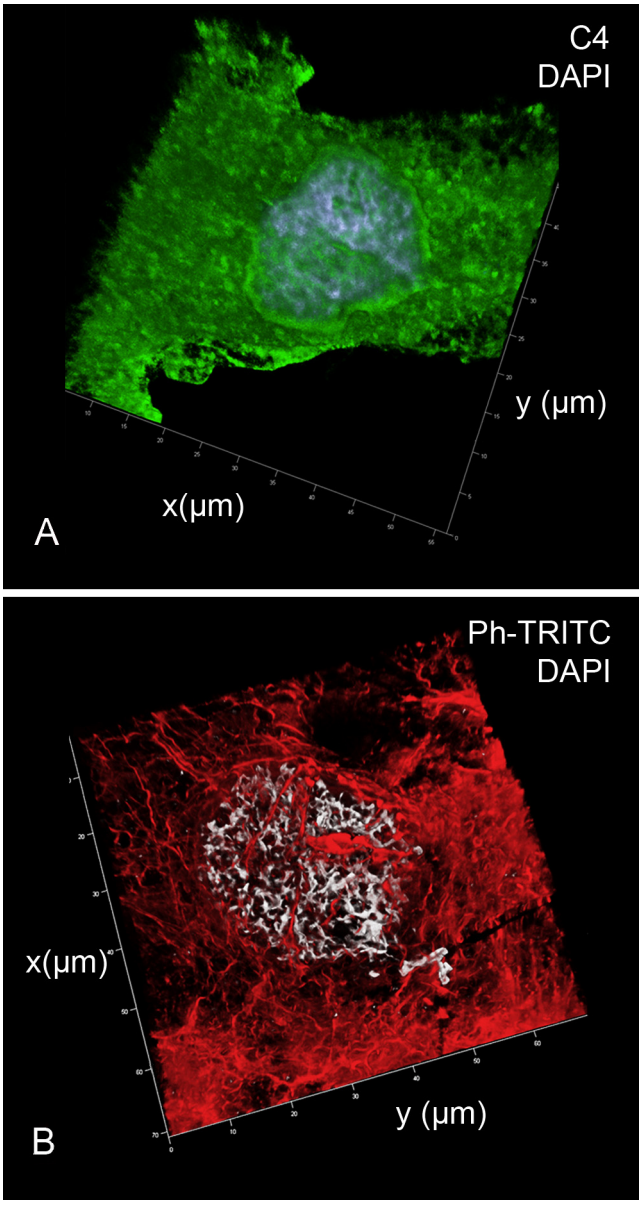

Fig. 4. 3D-reconstructions of actin distribution in the nucleus and adjacent cytoplasm after C4 anti-actin antibody labeling (A) and TRITC-phalloidin staining (B).

cal spatial organization of chromatin in the nucleus (Fig. 3B', B"). This suggests that latrunculin A treatment does not affect the general chromatin organization in *A. proteus*.

DNase I treatment

The predominant form of nuclear actin was tested by staining with fluorescent deoxyribonuclease I, which binds to unpolymerized actin. A clear staining of amoeba cells was observed (Fig. 5A). Both the dense nucleoplasmic actin meshwork and the continuous cytoplasmic peripheral actin layer were seen (Fig. 5A). An analysis of the merged images demonstrated almost complete co-localization of intranuclear actin (DNase I labeling) and chromatin strands (DAPI staining) (Fig. 5A', A"). The presence of the intranuclear meshwork and the peripheral layer surrounding the nucleus, both formed by G- and F-actin, were also demonstrable in confocal sections after double staining with DNase I and TRITC-phalloidin (Fig. 5B–B").

Discussion

Here we inspected the spatial distribution and forms of nuclear and cytoplasmic actin in *Amoeba proteus* (strain B) cells using immunocytochemical approaches. In the cells used in the study, the chromatin compartment demonstrated the organization that matched the interphase, prophase and prometaphase stages according to our previous observations (Demin et al. 2016). The shape of the intact amoeba nucleus reportedly varies from regular discoid to elongated ellipsoid or to irregular curved with small projections of the nuclear envelope, depending on the cell cycle stage (Demin et al. 2016). Intact nuclei possess a flattened shape and there was no significant effect on their profiles when the method of squashing was applied in this work.

According to recent discussions, nuclear actin could be present in all three forms – monomeric, oligomeric (short filaments that are not detected by phalloidin) and more rarely polymeric (Bettinger et al. 2004; Wesolowska and Lénárt 2015). Nuclear actin filaments are routinely reported, especially in the specialized cells such as oocytes (Parfenov et al. 1995; Świątek 1999; Rübsam and Büning 2001; Bogolyubov et al. 2013). However, it is still a matter of discussion whether oocyte nuclear actin constitutes canonical F-actin filaments or exists in a more specialized state (Gall 2006). In somatic cells, filamentous actin usually occurs upon stress or can be artificially induced under polymerization-related experimental conditions (Wesolowska and Lénárt 2015).

We used phalloidin staining to examine the presence of F-actin in the amoeba nucleus. No significant amount of F-actin was detected in the nucleus; instead, F-actin formed a cytoplasmic meshwork of filaments and bundles supporting the nucleus. As a result, the amoeba nucleus appears embraced by a basket-like structure formed by filamentous actin that is directly incorporated into a global cytoplasmic system of actin cytoskeleton (Fig. 4B). According to the analysis of cells labeled by fluorescent DNase I and anti-actin antibodies, actin monomers and oligomers form a dense layer adjacent to the cytoplasmic surface of the nuclear envelope (Figs 2, 4A, 5).

Our results confirm and significantly clarify the data on the organization and functioning of circumnuclear actin that were reported in previous works (Grębecka et al. 1999; Pomorski and Grębecka 1993, 1995; Wasik et al. 2000; Pomorski et al. 2007). Experiments with latrunculin A, carried out in *A. proteus* for the first time, have demonstrated that polymeric actin does not play an essential role as a structural scaffold for the inner space of the amoeba nucleus, although it can probably

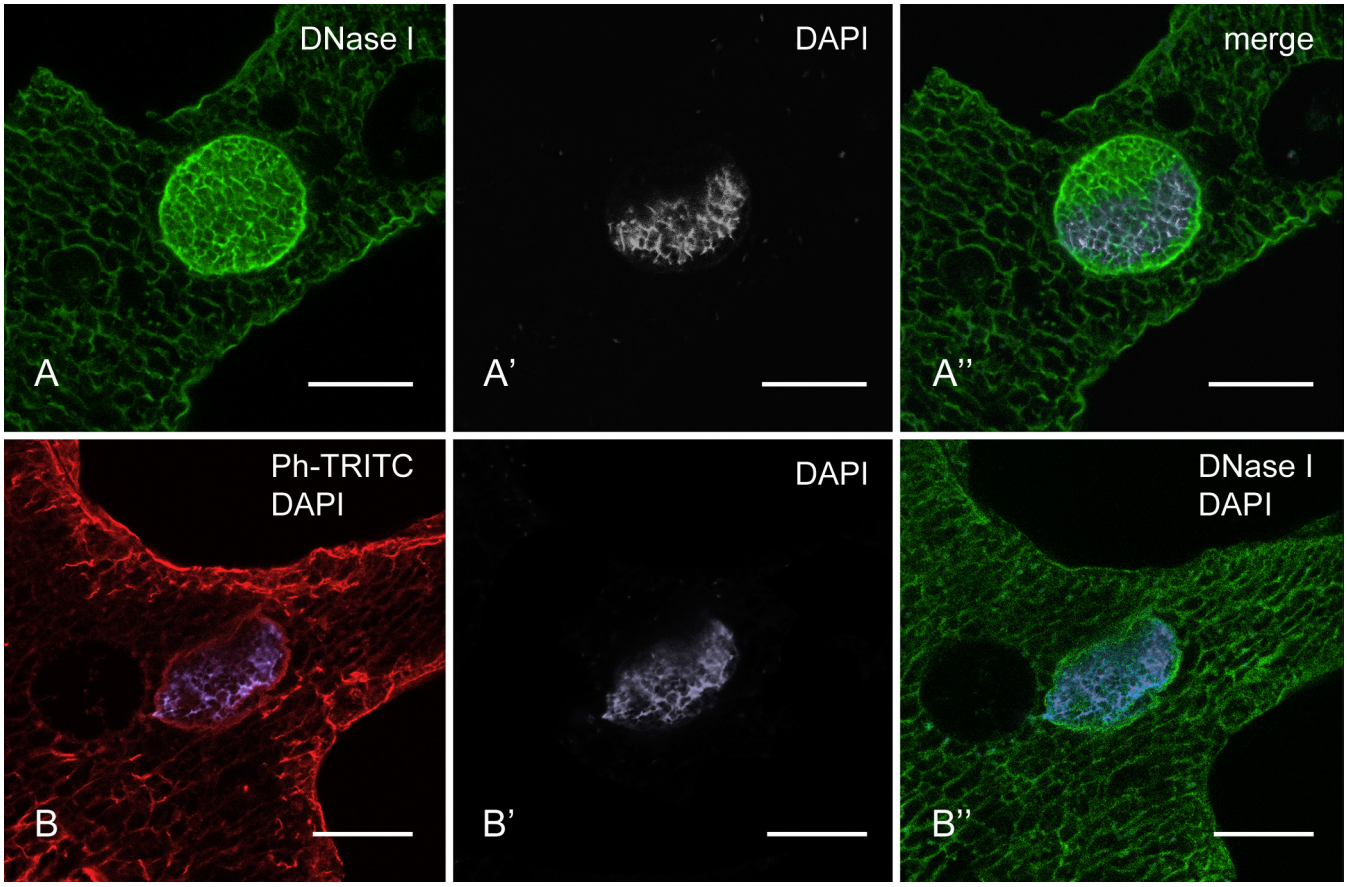

Fig. 5. Analysis of predominant forms of nuclear actin in *Amoeba proteus*. Single confocal sections through the XY plane of the nucleus after staining with fluorescent DNase I (A–A") and after double-staining with DNase I and TRITC-phalloidin (B–B"). Scale bars: 20 μm.

take a part in maintenance of the nuclear shape. The honeycomb layer underlying the inner membrane of the nuclear envelope in amoeba cells probably plays a nucleoskeletal role. It is the most prominent structure in the *A. proteus* nucleus, reaching 400–500 nm in thickness and consisting of a densely packed fibrous material organized into hexagonal prisms (Daniels 1973; Page 1986; Pappas 1956). The nature of the honeycomb layer is still unknown, and even the prevailing hypothesis that the honeycomb layer in *Amoeba* corresponds to the metazoan nuclear lamina, has not been confirmed (Batsios et al. 2012; Melcer et al. 2007; Schmidt et al. 1995).

Our fluorescent data showing that the nucleoplasm of *A. proteus* lacks a significant amount of F-actin contradict several earlier ultrastructural observations that suggested the presence of actin filaments in the amoeba nucleus (Grębecka et al. 1999; Gromov 1985). Intranuclear filaments could be due to fixation artifacts during sample preparation for electron microscopy or to other types of filaments in the nucleus (Wesolowska and Lénárt 2015). We conclude that the intranuclear actin pool is represented mostly by mono- and oligomers in *A. proteus* cells. It can be assumed with a high probability that rapid transition between monomeric and polymeric forms of actin occurs in the amoeba nuclear apparatus, and this may be crucial for facilitating nuclear export of RNP particles.

The spatial distribution and form of the intranuclear actin pool in *A. proteus* cells were elucidated in this work. Nuclear actin structures were found co-localized with chromatin. We observed similar patterns of actin distribution in all cases of indirect labeling of *A. proteus* cells with different primary antibodies. Certain differences in the character of fluorescence of intranuclear and cytoplasmic actin were previously reported for early mouse embryos labeled with antibodies against the N- and C-terminal domains of actin molecule (Bogolyubova and Bogolyubova 2009). Here the antibody against the C-terminal actin fragment revealed nuclear actin with higher efficiency, especially in areas of condensed chromatin, whereas the antibody to the N-terminus stained mostly the cytoplasm and the area of dispersed chromatin localization in the nucleus. However, we did not observe such differences of staining patterns in our material.

According to the results of fluorescent DNase I labeling, the predominant form of *A. proteus* nuclear actin is G-actin. The staining pattern after indirect labeling with anti-actin antibodies and after direct labeling with DNase I was similar. We found a dense meshwork formed by actin monomers and strongly co-localized with the chromatin strands in

the nucleoplasm of *A. proteus*. Such localization of actin molecules is consistent with the functions that are currently attributed to nuclear actin – in particular, the participation in chromatin-remodeling and chromatin-modifying complexes, involvement in transcription and its regulation as well as in the processes of nuclear export and intranuclear transport (Hofmann 2009; Visa and Percipalle 2010).

Data concerning the presence of intranuclear actin in protist cells are currently quite fragmentary, but several key papers should be mentioned. For instance, the formation of intranuclear microfilament bundles after DMSO treatment was shown for the slime mold *Dictyostelium* (Fukui 1978). The presence of actin as a non-histone chromatin-associated protein has been also assumed for this organism on the basis of electrophoretic analysis of protein from purified chromatin (Pederson 1977). The detailed analysis of the distribution and functioning of actin4 subfamily protein in *Paramecium tetraurelia* cells indicated the presence of this actin isoform in the macronucleus and involvement in micro- and macronuclear development (Sehring et al. 2010). Nuclear polymeric actin was reported in association with chromosomes and nucleoli for some other species of the Alveolata group, e.g., the dinoflagellate *Prorocentrum micans* and the ciliate *Paramecium caudatum* (Soyer-Gobillard et al. 1996; Benken and Sabaneyeva 2011). Nevertheless, we insist on the predominance of monomeric actin in the *A. proteus* nucleus. In light of the mentioned works it seems reasonable to examine the co-localization of actin with nucleoli in the amoeba nuclei. Some differences in organization of the nuclear actin pool among protists from distant groups seems possible, but should be suggested with great caution.

In conclusion, our data on the spatial organization of nuclear actin in *A. proteus* is consistent with recent findings about the presence and role of different actin forms in the cell nucleus. Moreover, in the light of the fragmented information about nucleus-associated actin among unicellular eukaryotes, our results provide a better understanding of nuclear actin and its spatial organization in lower eukaryotic cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejop.2016.09.002.

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