Original

Distribution of Actin-like Proteins in the Ciliate *Spirostomum ambiguum*

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SUMMARY

Previous studies on cell contraction in the ciliate *Spirostomum* have suggested that actin may not be present, in spite of its ubiquity in other ciliates including *Paramecium* and *Tetrahymena*. In this study, different anti-actin antibodies were used to re-examine whether actin occurs in *Spirostomum*. Western blotting analysis with these anti-actin antibodies showed that actin-like proteins may exist in *Spirostomum*. Intracellular localization of actin-like proteins was examined by immunofluorescence microscopy with the same antibodies. Fluorescent signals were seen at the cleavage furrow, around the macronucleus, and at the ciliary bases including those of the membranelle. These results suggest that actin may exist in *Spirostomum*, but it is different from skeletal muscle actin and *Tetrahymena* actin in antigenic characteristics.

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INTRODUCTION

The large heterotrichous ciliate *Spirostomum* is known for its remarkable rapid cell contraction that is triggered by external stimuli, and the cytoskeleton-based motile systems of contraction and subsequent re-elongation have been studied in order to understand its underlying molecular machineries (Yogosawa-Ohara et al., 1985; Ishida et al., 1988). The possible involvement of actin in cell contraction was investigated by Valeri et al. (1983), who demonstrated by transmission electron microscopy that myosin S-1 from rabbit skeletal muscle did not form its characteristic "arrowhead" complex in the cortex of *Spirostomum*. They further showed no DNase-I inhibition activity by *Spirostomum* cell homogenate, and concluded that actin may not occur in *Spirostomum*. Based on these results, it has been surmised that contraction in *Spirostomum* does not involve actin.

In *Tetrahymena*, Yasuda et al. (1980) and

Jerka-Dziadose (1981) showed that dividing cells have actin filaments as a component of the contractile ring structure. *Tetrahymena* actin shares fundamental characteristics with other actin proteins, including polymerization activity and interaction with myosin, but it does not bind to phalloidin, DNase-I and α-actinin (Hirono et al. 1989), indicating that protozoan actin may be different from vertebrate actins. In view of these facts, we have re-examined the possible existence of actin in *Spirostomum* using biochemical and immunohistological techniques.

MATERIALS AND METHODS

Culture

Spirostomum ambiguum was used in this study. *Spirostomum* cells were cultured at 22°C in ×100 diluted Knop's solution (0.24 mM $Ca(NO_3)_2$, 0.14 mM KNO_3, 0.06 mM $MgSO_4$ and 0.1 mM KH_2PO_4) supplemented with boiled wheat grains. Fresh culture solution and boiled wheat were added to the medium every two weeks. *Tetrahymena thermophila* was cultured according to the method of Hirono et al. (1987a). Cells were harvested at the logarithmic phase of growth.

Electrophoresis

After the cells were washed thoroughly, they were collected into a small test tube and homogenized. Proteins were solubilized with 8 M urea and centrifuged at 4,000 × g for 5 min, and the supernatant was dialyzed against 10 mM Tris-HCl buffer (pH 7.2) to remove urea. The dialyzed supernatant was used as the specimen for electrophoresis. As control samples for comparison, *Tetrahymena* proteins and crude extract of chicken skeletal muscle were prepared by the same procedure. SDS polyacrylamide-gel electrophoresis (SDS-PAGE) was performed following Laemmli's method (Laemmli 1970) with 3% stacking and 8% separating gels. Gels were stained with Coomassie brilliant blue.

Immunoblotting

The specimens of *Spirostomum*, *Tetrahymena* and chicken skeletal muscle were electrophoresed and blotted onto a polyvinylidene fluoride (PVDF) membrane. The blotted PVDF membranes were blocked with 5% skim milk in TBS (140 mM NaCl, 50 mM Tris HCl, pH 7.4) for 60 min at room temperature (RT) and incubated with ×100 diluted primary antibodies for 1 h at RT. After the treated membranes were washed with TBS and TBS-Tween, they were reacted with ×1,000 diluted secondary antibodies for 1 h at RT. After being washed, they were stained with diaminobenzidine.

Antibodies

Primary antibodies used in this study were: a rabbit polyclonal IgG against 11 amino acid residues at the C-terminal of vertebrate skeletal muscle actin (antibody-I, Sigma A2066), a rabbit anti-chicken skeletal muscle actin antiserum (antibody-II, Sigma A2668), and a rabbit polyclonal antibody raised against the N-terminal portion of *Tetrahymena* actin (Hirono et al. 1987a; antibody-III).

Immunofluorescence staining

Dividing cells were chosen from culture dishes in which cells were in the logarithmic growth phase, and were washed with fresh culture medium. The cells were then treated for immunofluorescence staining by the method of Hirono et al. (1987b). The primary antibodies were the same as in the immunoblotting. FITC-labeled anti-rabbit goat IgG was used as a secondary antibody.

Phalloidin staining

Air-dried cells prepared as for the immunostaining were fixed in Carnoy's fixative (ethanol: chloroform:acetic acid = 6:3:1) for 20 min. After being washed with ethanol and PBS, the cells were

processed for membrane permeabilization with 1% Triton-X100 in PBS, and then washed with PBS. They were reacted with rhodamine-tagged phalloidin for 20 min, then washed, and examined under a fluorescence microscope.

RESULTS AND DISCUSSION

Actin plays an essential part in cell movement in a wide range of species, from protozoans to higher animals and plants. It is a highly conserved protein and its primary structure shows remarkably high homology among different species. However, it is reported that actins in some ciliates like *Tetrahymena* and *Paramecium* show low homology to other actins in their amino acid sequence (Hirono et al., 1987a, 1990; Diaz-Ramos et al., 1998). To detect actin in *Spirostomum*, Valeri et at. (1983) applied biochemical procedures that were developed for detecting vertebrate actins. Such procedures may not be appropriate for protozoan species, and this might be the reason they failed to detect actin in *Spirostomum*.

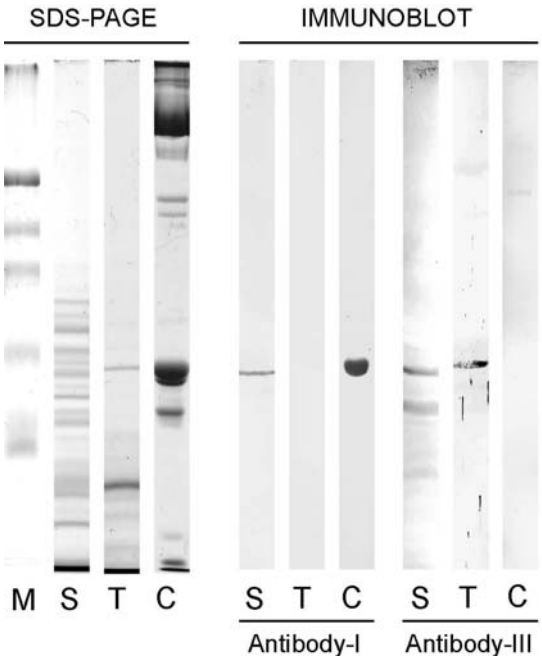

Fig. 1. SDS-PAGE of the extract of *Spirostomum* cells and immunoblots with antibody-I and antibody-III. M; standard molecular markers (116, 84, 58, 45 and 26.5 kDa), S; *Spirostomum* cell extract, T; *Tetrahymena* cell extract, C; crude extract of chicken skeletal muscle. Antibody-I; immunoblots showing the distribution of actin-like proteins detected by antibody-I, Antibody-III; immunoblots detected by antibody-III.

Immunoblotting

Antibody-I specifically stained a single band of 40.5 kDa in an extract of *Spirostomum* (Fig 1). This antibody scarcely stained *Tetrahymena* actin. Antibody-II did not react with protozoan cell extracts, but exclusively reacted with chicken skeletal muscle. Antibody-III stained a band of 40.5 kDa in *Spirostomum* as with antibody-I. However, antibody-III gave weaker staining than antibody-I, and an additional 27-kDa band was also stained. Antibody-III did not detect chicken skeletal muscle actin. These findings show that antigens for anti-actin antibodies exist in *Spirostomum* cell extract. In particular, the fact that antibody-I detected a single band with high specificity strongly suggests the existence of an actin-like protein with molecular weight of about 40.5 kDa in *Spirostomum*. This protein cross-reacted with an anti-*Tetrahymena* actin antibody, indicating that the protein has epitopes binding to both anti-vertebrate actin and anti-*Tetrahymena* actin antibodies. Since skeletal muscle actin does not cross-react with the anti-*Tetrahymena* actin antibody (antibody III), the actin-like protein in *Spirostomum* may have a unique amino acid sequence that is different from both skeletal muscle and *Tetrahymena* actins.

Phalloidin staining

Fluorescence microscopy after rhodamine-phalloidin staining failed to show any fluorescent structure in *Spirostomum* (data not shown here). It has been reported that *Tetrahymena* actin cannot be stained with phalloidin because *Tetrahymena* actin

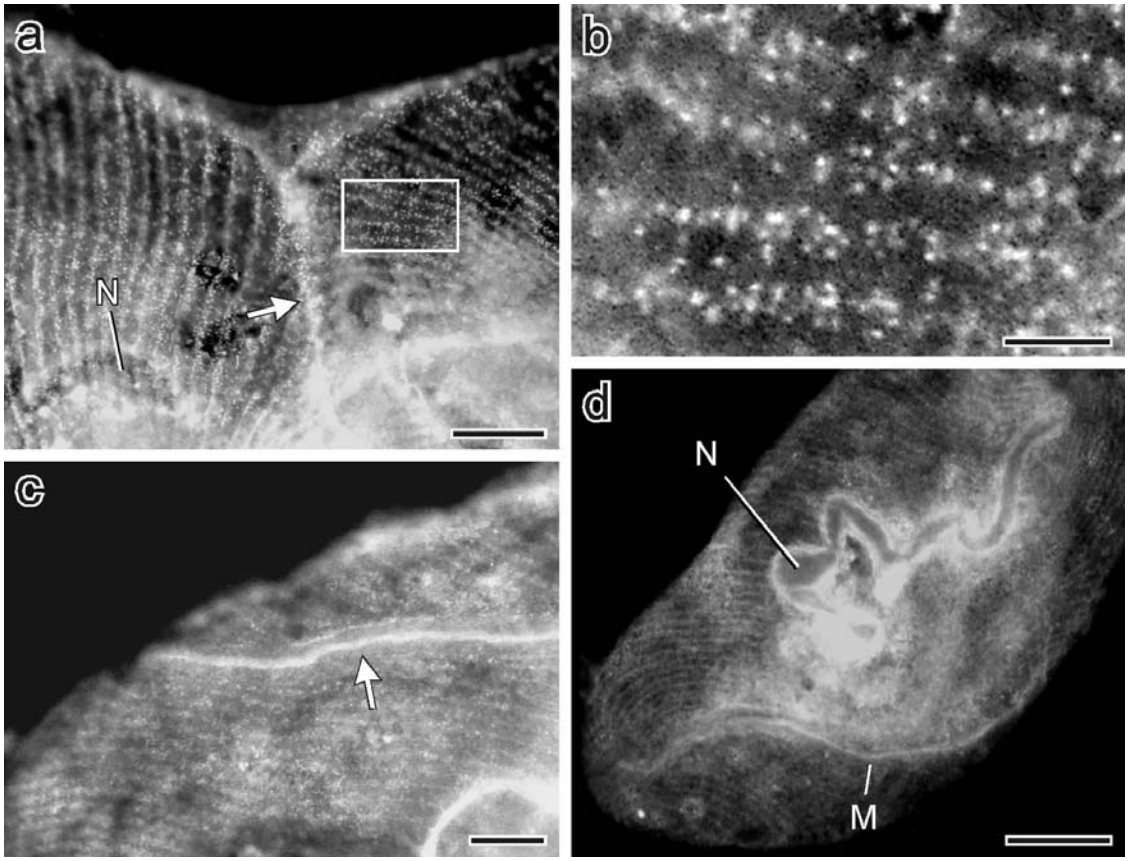

Fig. 2. Immunofluorescence micrographs of *Spirostomum ambiguum* stained with antibody-I (a-c) or antibody-III (d). In the middle part of a dividing cell (a), a line of bright fluorescence is present at the division furrow (shown by an arrow). At the cell surface, fluorescence signals are localized as small dots aligned along the ciliary lines, which are clearly seen in an enlarged picture (b) of the area shown by a rectangle in (a). Materials around the macronucleus (N in a and d) and along the membranelle (arrow in c and M in d) were also stained. Scale bars: 20 μm (a), 5 μm (b), 20 μm (c) and 50 μm (d).

lacks the binding site for phalloidin (Hirono et al., 1989). The *Spirostomum* actin-like protein may also lack the phalloidin binding site.

Immunofluorescence microscopy

When *Spirostomum* was stained with antibody-I, fluorescent signals were detected at the periphery of the macronucleus, at the cell surface as dots distributed along the ciliary line, and along the membranelle (Fig. 2). In cells undergoing division, ring-shaped fluorescence was observed at the division furrow (arrow in Fig. 2a). The same patterns of antibody staining were obtained when cells were stained with antibody-III, although staining was weaker than with antibody-I (Fig. 2d). As in the immunoblotting experiment, almost no fluorescence was found in *Spirostomum* when it was stained with antibody-II.

In *Tetrahymena*, antibody-III has been reported to recognize cortical rows of basal bodies, division furrows, intracellular filaments, a part of the oral structure, food vacuoles and contractile vacuole pores (Hirono et al., 1987a, b). The specific staining at the division furrow in *Spirostomum* suggests the possibility that actin may be involved in cytokinesis. It may also correspond to the filamentous

ring-like structure observed under the division furrow of *Stentor coeruleus*, a heterotrichous ciliate closely related to *Spirostomum* (Diener et al., 1983). The spotty localization of antibody-binding structures on the cell surface along ciliary lines and the membranelle suggests that at least some, if not all, of the fluorescent dots may correspond to basal bodies. In *Tetrahymena*, the ciliary basal body is known to contain actin as one of its constituent proteins (Hirono, 1987b; Hoey and Gavin, 1992). In contrast to *Tetrahymena*, the dot-like structures on the cell surface of *Spirostomum* are not aligned in a straight line, but are located in bands that lie along ciliary lines. They are apparently more numerous than the basal bodies, indicating that only some of the dots can be attributed to basal bodies. Further studies with electron microscopy are needed to understand the precise localization of the actin-like proteins in *Spirostomum*.

In summary, this study demonstrated actin-like proteins in *Spirostomum* which had previously been surmised to contain no actin. These proteins may be different from actins in higher animals and *Tetrahymena* in both molecular weight and antigenicity.

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