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Actin and major sperm protein in spermatozoa of a nematode, *Graphidium strigosum* (Strongylida: Trichostrongylidae)

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Abstract. In most amoeboid cells, the main protein involved in motility is actin. Nematode sperm are an exception, and their amoeboid motility is based on major sperm protein (MSP). We have studied the localization of actin and MSP in spermatids and spermatozoa of *Graphidium strigosum* (Dujardin, 1845), a species which has elongate male germ cells in which organelles are easily identified. Electrophoreses of *G. strigosum* sperm proteins indicate that the main protein band, about 15 kDa in molecular weight, is specifically recognized by an anti-MSP polyclonal antibody developed against MSP of *Caenorhabditis elegans* (Burke and Ward 1983). Actin is present in small quantities. Immunocytochemical observations reveal that actin and MSP have an identical localization in precise areas of the male germ cells. Spermatids are labelled as dots around a central unlabelled zone, and spermatozoa are labelled only at the level of the anterior cap. Observations in *G. strigosum* are similar to that previously obtained in *Heligmosomoides polygyrus* (Mansir and Justine 1996). Co-localization of actin and MSP in the anterior cap of the spermatozoon, the region associated with pseudopod production, does not demonstrate directly that actin is involved in amoeboid movements, but shows that the role of actin in the cytoskeleton of nematode sperm should be re-investigated.

Amoeboid cell motility plays an important role in diverse biological processes such as cell migration. In most cases, the propulsive force is generated by an actin-myosin based set of chemomechanical proteins (Oliver et al. 1994)

One exception is the crawling sperm of nematodes which moves by extending a pseudopod that undergoes localized protrusion, membrane ruffling, and cytoskeletal flow, and in which the role usually assigned to actin has been taken over by major sperm protein (MSP). This substitution provides a unique opportunity for comparing pseudopod behaviour based on different proteins (Italiano et al. 1996, Theriot 1996, Roberts and Stewart 1997). MSP, a sperm-specific protein, accounts for 10 to 15% of nematode sperm protein (Klass et al. 1982). It forms a system of filaments organized into branching fibres that packs the pseudopod and is stated to act in the absence of actin (Sepsenwol et al. 1989, Scott 1996, Theriot 1996). Nematode sperm undergo locomotion by polymerizing MSP at the distal perimeter of the pseudopod and depolymerizing MSP at the junction of the pseudopod and cell body (Sepsenwol and Taft 1990, Roberts and King 1991). Several studies on *Ascaris suum* and *Caenorhabditis elegans* (Nelson and Ward 1981, Nelson et al. 1982, Roberts and Streitmatter 1984) have shown that actin is a minor cytoskeletal protein in the sperm, respectively less than 0.5% and 0.02% of the proteins, in contrast to other amoeboid cells in which actin represents 5-20%.

We have recently studied spermatozoa in three species of trichostrongyle nematodes, *Heligmosomoides polygyrus*, *Trichostrongylus colubriformis* and *Teladorsagia circumcincta*. These species have large elongated spermatids and spermatozoa in which visualization of cell organelles and precise localisation of the cytoskeletal proteins can be easily made (Mansir and Justine 1995, 1996, 1998, Mansir et al. 1997). An important and unexpected result was the co-localization of actin and MSP in spermatids and spermatozoa of these species.

In this paper, a comparative study has been undertaken in *Graphidium strigosum* (Dujardin, 1845), which also has elongated spermatozoa. Immunocytochemical observations showed that actin and MSP are colocalized in precise regions of the cell, thus confirming results obtained on other nematode species.

MATERIALS AND METHODS

Specimens of *Graphidium strigosum* (Dujardin, 1845) were collected from the stomach of naturally infested wild rabbits, *Oryctolagus cuniculus* (Linnaeus, 1758), from Versailles, France.

Antibodies used for immunoblots and immunocytochemistry. Actin was detected with a monoclonal antibody (N350) developed against chicken gizzard actin (Amersham). Major-sperm-protein was detected with a polyclonal antibody against MSP of *Caenorhabditis elegans* (Maupas, 1899) (Burke and Ward 1983), generously provided by Prof. Samuel Ward. The same antibodies were used for immunoblots and immunocytochemistry.

Analysis of proteins in germ cells. Two protein extracts were prepared, from male germ cells (gonia to spermatozoa),

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and from female bodies. The female body extract was prepared from 80 adult females; only the anterior part of each body was kept, to avoid contamination by spermatozoa in the posterior female organs of inseminated specimens. The male germ cell extract was prepared from 80 adult males. The genital system of each worm was individually dissected as described previously (Mansir and Justine 1996). Lysis was performed in water for actin experiments and in Tris buffer (0.02M, pH 7.6) with EDTA and beta-mercaptol for MSP experiments. The lysates were centrifuged at 100 000 g for one hr in a Beckman XL-70 ultracentrifuge. Proteins of the supernatant were concentrated on a filter (Microconcentrator Centricon-10 Amicon) for 1-3 hr at 5000 g, and stored at -70°C.

SDS-PAGE electrophoreses were performed with a 15% gel (Laemmli 1970). A prestained low molecular weight kit (Bio-Rad, 5 µl) was run simultaneously. For each sample, two gels were run simultaneously (Bio-Rad Mini-Protean II Dual Stab). One was stained (Coomassie Brilliant Blue R-250) and the other was used for the immunoblot.

For immunoblots, proteins were transferred onto a nitrocellulose membrane (Hybond-C, Amersham). The membrane was then saturated with 5% skimmed milk overnight at 4°C. After washing with TBS (Tris Buffer Saline), 3 x 10 min, the membrane was bathed in the antibody. We used a monoclonal anti-actin, 1/1000 in TBS, or a polyclonal anti-MSP antibody, 1/1600 in TBS, 2 hrs at room temperature. After washing (PBS, 3 × 10 min) the second antibody (respectively Goat anti-mouse or Goat anti-rabbit conjugated with alkaline phosphatase, Nordic, 1/15000) was applied for 2 hrs at room temperature. The labelling was revealed in Nitroblue Tetrazolium (Sigma, 50 mg/ml, 0.67%, 5-15 min) and 5-Bromo-4-Chloro-3-Indoyl phosphate (Sigma, 50 mg/ml), and the membrane was then rinsed in revelation buffer (Tris 0.1M, NaCl 0.1M, $MgCl_2$ 5mM), photographed, and dried.

Interference contrast light microscopy. Male germ cells, obtained by dissecting adult male worms in a drop of salt water (NaCl 9‰), were observed in salt water between a slide and a cover glass, with an Olympus BH-2 light microscope equipped with Nomarski interference contrast.

Immunocytochemistry. Germ cells were obtained by dissecting each male in a drop of PBS (phosphate buffer saline, Sigma) on a pit slide (Mansir and Justine 1996) and cells were allowed to sink and adhere to the slide (1 hr), then fixed with 3.7% formaldehyde in PBS (15 min) and finally permeabilized (15 min) in 0.1% Triton X-100 in PBS or in 3% SDS in PBS. Non-specific antigenic sites were blocked with 2% Bovine Serum Albumin (Sigma) in PBS (BSA-PBS) for 45-90 min at room temperature. Antibodies (monoclonal anti-actin antibody, 1/100, 1/10; polyclonal anti-MSP antibody, 1/1600) were applied in BSA-PBS for 40 min at room temperature. After washing (PBS 3 × 5 min) the FITC-conjugated antibody (Goat anti-mouse or Goat anti-rabbit according to the first antibody, Nordic, 1/40 in PBS) was applied for 40 min at room temperature and then washed (PBS 3 × 5 min). Propidium Iodine (100 µg/ml in PBS, 10 min) was applied for the labelling of nuclei. After a wash (PBS 3 × 5 min), mounting was done in Citifluor (Citifluor Ltd, London, UK) and slides were sealed with nail enamel. Controls were made by omitting the first antibody; they were negative and thus are neither commented on nor illustrated. Observations were made with a Nikon Optiphot epifluorescence microscope equipped with filters for FITC channel (B-2A) and TRITC channel (G-2A).

RESULTS

Protein analysis Fig. 1

Male germ cell samples (Fig. 1a) showed numerous protein bands with a 15 kDa major band. A strong reactivity was seen in the immunoblot with anti-MSP polyclonal antibody on the same band (Fig. 1b), suggesting large amounts of MSP in male germ cells of *Graphidium strigosum*. A single faint band at nearly 43 kDa was detected by anti-actin monoclonal antibody in the immunoblot (Fig. 1c). Thus, the presence of actin, in small quantities, was demonstrated. An electrophoresis of female proteins was performed as a negative control to assess the specificity of the labelling. The electrophoresis of total female protein (Fig. 1d) showed the absence of the male major band at 15 kDa, and no labelling was detected in the immunoblot with the anti-MSP polyclonal antibody (Fig. 1e).

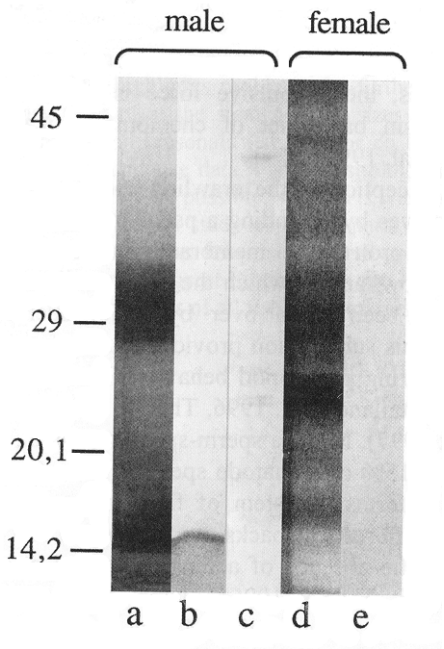

Fig. 1. Western blots of *Graphidium strigosum*. Lanes a-c, protein extracts from male germ cells; lanes d-e, crude homogenates of anterior parts of female bodies. **a**, **d** – Coomassie blue stained lane. An abundant 15 kDa band, corresponding to major sperm protein (MSP) is visible for male cells, but not for female bodies. **c** – Actin is detected in small quantities in male germ cells. **b**, **e** – Immunostaining with anti-major sperm protein (MSP). MSP is the major band in male germ cells and is absent in female bodies. Note that the two antibodies (anti-actin and anti-MSP) do not cross-react.

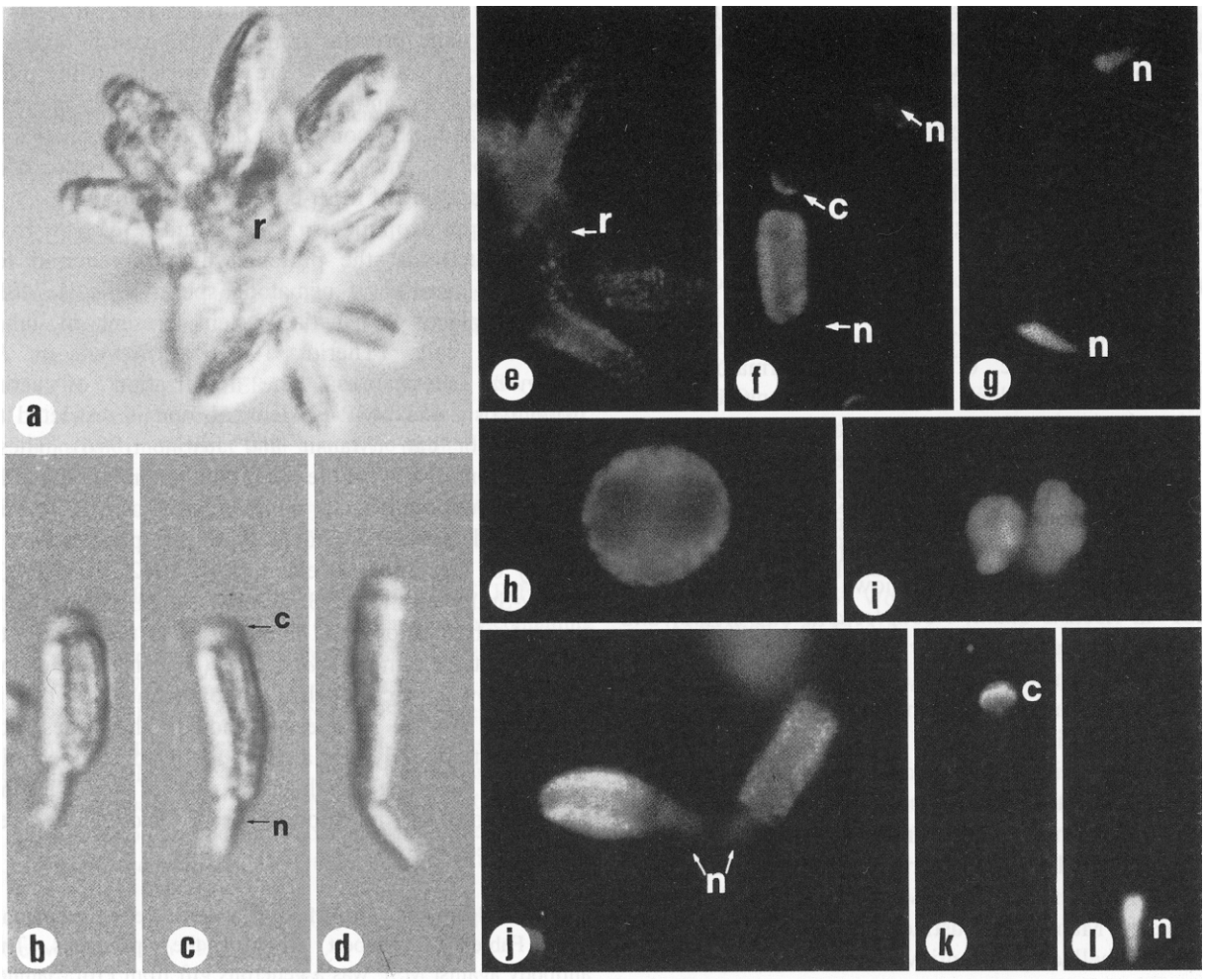

Fig. 2. Male germ cells of *Graphidium strigosum*. **a-d** – Interference contrast. **a –** Spermatids attached to rachis. **b**, **c** – Spermatids separated from rachis. **d** – Spermatozoon. Note posterior cone-shaped nucleus and anterior bulbous cap. **e-g –** Actin labelling. **e** – Spermatids attached to rachis, anti-actin labelling. **f** – Contrasting actin labelling of spermatid and spermatozoa. The spermatid has a peripheral region heavily labelled and central core unlabelled, but spermatozoon is labelled only at level of anterior cap. **g** – Nuclear labelling corresponding to f. **h-l** – MSP labelling. **h** – Round cells in mitosis, probably spermatocytes. MSP labelling in the cytoplasm. **i** – Nuclear labelling corresponding to h. **j** – Spermatids attached to rachis; left, focus on the centre of cell, peripheral region heavily labelled and central core unlabelled; right, focus on surface, labelling as dots. **k** – Spermatozoon. MSP labelling strictly restricted to anterior cap. **l** – Nuclear labelling corresponding to k. a-d, ×3000; e-l, ×2200. c – anterior cap; n – nucleus; r – rachis.

These results show that male germ cells of *G. strigosum* contain small quantities of actin and large quantities of MSP, and that MSP is present in male only. No cross-reactivity between the anti-actin and anti-MSP antibodies was detected, thus validating the immuno-fluorescence results.

Morphology of male germ cells and immunolabelling

Fig. 2

Early spermatids had an elongated shape and were attached to a rachis (Fig. 2a). More advanced cells were free from the rachis (Fig. 2b, c). The spermatozoa were thinner and more elongated cells (Fig. 2d). Spermatids and spermatozoa were characterized by a pyriform nucleus in the posterior part and an elongate cell body showing an anterior bulbous region or "cap".

The immunolabelling with anti-actin monoclonal antibody showed very different patterns of labelling in the two cell types (Fig. 2e-g). In early spermatids, linked to the rachis, the staining was limited to the periphery of spermatids, whereas the central core and the anterior cytoplasmic region, opposite the nucleus, was free from labelling (Fig. 2e, f). In spermatozoa, the labelling was localized in the anterior region, in the form of a very thin crescent-shaped zone (Fig. 2f).

The immunolabelling with anti-MSP antibody in spermatids (Fig. 2j) was identical to that obtained with anti-actin antibody. In spermatozoa (Fig. 2k), the labelling was similar to the actin labelling but, instead of being restricted to the anterior region of the cap, occupied all the cap region. A dot-like labelling of the cytoplasm was also visible in early stages of spermatogenesis, such as dividing spermatocytes (Fig. 2h).

DISCUSSION

Spermatozoa of *Graphidium strigosum* show the usual characteristic of nematode spermatozoa, i.e. absence of a flagellum, and they are elongated with a strong antero-posterior differentiation, a feature found in several nematodes, and particularly in the trichostrongyles (Mansir and Justine 1996).

The antibody against MSP from *Caenorhabditis elegans* (Roberts et al. 1986, Ward et al. 1986) used in the present study has been shown to label selectively MSP in *Ascaris suum*, *Neoaplectana intermedia*, and *Heligmosomoides polygyrus* (Hess and Poinar 1989, Sepsenwol et al. 1989, Mansir and Justine 1996). The wide species range of this antibody confirms the strong molecular similarities of MSP in various nematodes, also apparent in term of aminoacids sequences (Scott 1996). The absence of cross-reactivity with actin validates the immunocytochemical observations.

In a detailed study of spermatids and spermatozoa of *H. polygyrus* involving SEM, TEM and immunocytochemistry, Mansir and Justine (1996) demonstrated that the dots labelled by the anti-MSP antibody in spermatids were the fibrous bodies, whereas the anterior cap corresponded with a region occupied with a network of MSP fibres in the mature spermatozoa. Actin was found with the same localization. Actin was also detected in the fibrous bodies of spermatids of a primitive nematode, *Sphaerolaimus hirsutus* (Noury-Sraïri et al. 1993). It has been demonstrated in *C. elegans* that the fibrous bodies are organelles specialized for the storage of MSP in spermatids (Ward and Klass 1982, Roberts et al. 1986). The present immunocytochemical observa-tions on *G. strigosum* are consistent with these observations, and in this species, we can safely interpret the actin- and MSP-containing dots in the spermatids as fibrous bodies, and the anterior cap of spermatozoa as chiefly consisting of MSP-based cytoskeleton.

Studies on two models, *A. suum* and *C. elegans* have demonstrated that the protein which is required for amoeboid motility of nematode sperm cells is MSP, which replaces the actin system present in other amoeboid cells (Theriot 1996). However, in *H. polygyrus* spermatozoa, the localization of actin, tropomyosin and MSP is identical, and is restricted to the anterior cap (Mansir and Justine 1996). These observations challenge the prevailing theory about MSP, which is supposed to act in the absence of actin. Actin has been demonstrated, however, in the spermatids of a variety of nematodes (Nelson et al. 1982, Foor 1983, Noury-Sraïri et al. 1993, Mansir et al. 1997). Our present observations in *G. strigosum*, together with results obtained in *H. polygyrus* (Mansir and Justine 1996) show that co-localization of actin and MSP is found in several species. Although this does not demonstrate directly that actin is involved in amoeboid movement, it suggests that the role of actin in the cytoskeleton of nematode sperm should be re-investigated.

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