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Identification and localisation of a putative actin in the two species of potato cyst nematodes using a monoclonal antibody

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Summary – A monoclonal antibody (MAb RES GRj-2) raised to second stage juveniles (J2) of *Globodera rostochiensis* binds to the layer of somatic muscle in J2 of the two species of potato cyst nematodes (PCN). MAb binding was not observed in gravid white females. The MAb recognised a protein of 42 kDa which is similar to mammalian actin, since the MAb also reacted with bovine muscle actin in Western blots. Commercial polyclonal antiserum to actin from chicken back muscle also bound to a band of 42 kDa in extracts of *G. rostochiensis* and *G. pallida* and to a commercial sample of actin. The isoelectric points of the proteins recognised by the MAb were pI 5.72 in *G. rostochiensis* and bovine muscle actin, and pI 5.46 in *G. pallida,* indicating that this putative actin has different isoforms in the two species of PCN.

Résumé – ***Identification et localisation de l'actine dans les deux espèces de nématodes à kystes de la pomme de terre grâce à des anticorps monoclonaux*** - Il est montré que l'anticorps monoclonal (AcMc RES GRj-2) produit contre des juvéniles de stade II (J2) de *Globodera rostochiensis* colore la couche de muscle somatique des J2 chez les deux espèces de nématodes à kystes de la pomme de terre (PCN). La réaction est spécifique aux J2 et n'apparaît pas chez les femelles blanches gravides. Cet AcMc reconnaît une protéine de 42 kDa qui présente des similitudes avec l'actine des mammifères, puisqu'il réagit également avec l'actine musculaire bovine en « Western blot ». Des anticorps polyclonaux produits contre l'actine des muscles du dos de poulet réagissent avec une protéine de même poids moléculaire de 42 kDa dans les extraits de J2 de *G. rostochiensis* et *G. pallida* et dans un échantillon commercial d'actine. Les points isoélectriques des protéines reconnues par le AcMc varient légèrement entre les deux espèces de PCN et l'actine. Ils sont approximativement de 5,72 chez *G. rostochiensis* et pour l'actine musculaire bovine et de 5,46 chez *G. pallida,* indiquant que l'actine présente des isoformes différentes chez les deux espèces de PCN.

Key words : actin, *Globodera rostochiensis, G. pallida,* immunolocalisation, monoclonal antibody, somatic, muscle, nematodes.

Characterisation of proteins and genes from plant parasitic nematodes that are involved in vital processes such as locomotion, sensory perception, feeding and reproduction will provide the necessary knowledge and understanding on which to base the design of specific agonists/antagonists and new control measures. Monoclonal antibodies (MAbs) are an invaluable tool for the identification and characterisation of proteins and may help to unravel the specific physiological role of key molecules in host parasite-relationships (Hussey, 1989).

We have raised MAbs to a crude homogenate of second stage juveniles (J2) of *Globodera rostochiensis.* Antibodies showing differential recognition of the two species of potato cyst nematodes (PCN) *G. rostochiensis* and *G. pallida* were used to characterise and localise the antigens they recognise (Curtis & Evans, 1994). The MAb RES GRj-2 reacting with somatic muscle of J2 and recognising an antigen with a molecular weight similar to actin was chosen for further characterisation. Since actin is a well conserved protein (Schwob & Martin, 1992), it was of interest to investigate the reason for the differential recognition of the species.

Actin has been characterised in various organisms such as *Dictyostelium discoideum, Caenorhabditis elegans, Naegleria gruberi, Candida albicans, Toxoplasma gondii, Schistosoma mansoni, Onchocerca volvulus* (Eckert & Lazzarides, 1978; Files *et al.*, 1983; Fulton *et al.*, 1986; Fiss & Buckley, 1987; MacGregor & Shore, 1990; Zeng & Donelson, 1992). Four actin genes have been isolated from *C. elegans,* and it seems that the number of actin genes generally increases with the complexity of the organism (Files *et al.*, 1983). Actin filaments, in association with a family of myosin proteins, are required for cellular motile processes as diverse as vesicle transport, cell locomotion and cytokinesis, and also serve as structural support and components of membranes (MacGregor & Shore, 1990; Lees-Miller *et al.*, 1992; Clark & Meyer, 1992).

This study reports the presence of actin in the somatic muscle of the two species of PCN, using a monoclonal antibody. The antibody showed a differential recognition of the two species of PCN and here we show that a putative actin is antigenically different in the two species of PCN and that the antibody recognised different isoforms of the actin.

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Materials and methods

Antigen preparation

The nematode populations used were *Globodera rostochiensis* pathotype Rol from Woburn and *G. pallida* pathotype Pa2/3 from Cadishead. Antigens used for the immunisation of mice were prepared by homogenisation of hatched J2 of *G. rostochiensis* as described below. Otherwise, cysts of *G. rostochiensis, G. pallida, Heterodera avenae, H. goettingiana* and *H. schachtii* were used to prepare antigen by homogenisation in 0.1 M PBS (phosphate buffered saline) pH 7.2 containing 1 mN N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 1 mM N-tosyl-L-lysine chloromethyl ketone (TLCK), 1 mM phenylmethanesulphonyl fluoride (PMSF), 2 mM ethylenediaminetetra-acetic acid (EDTA) using a plastic homogenizer (Biomedix). In addition, *Globodera rostochiensis* and *G. pallida* cyst homogenates were also prepared by adding 1 % sodium deoxycholate (DOC) to the above solution, in order to improve the efficiency with which membrane-bound proteins were extracted. Homogenization was conducted on ice and the homogenate checked under the microscope periodically to ensure that maximum disruption of cuticles had occurred. The supernatants were recovered after centrifugation using a microfuge and used immediately or stored at – 20 °C until needed. Protein concentrations were measured by the method described by Bradford (1976).

Monoclonal antibody production

A Balb/c mouse was immunized three times, at 3-week intervals, intraperitoneally with 100 µg of *G. rostochiensis* J2 homogenates emulsified in an equal volume of Freund's complete adjuvant for the first injection only. A fourth intraperitoneal booster injection of 100 µg was given 3 days before fusion. MAbs were prepared using standard protocol (Galfre & Milstein, 1981).

Positive hybridomas were screened for reactivity against homogenate of the two species of PCN using indirect enzyme-linked-immunosorbent assay (ELISA). MAbs showing differential recognition of the two species of PCN were cloned twice by limiting dilution (Curtis & Evans, 1994). Immunoglobulin class and subclass identity of the MAbs was determined by a double immunodiffusion technique (Ouchterlony, 1958) using antisera directed against the mouse Ig heavy chains (anti-mouse IgA, IgM, IgG1, IgG2a, IgG2b and IgG2c) supplied by ICN ImmunoBiologicals.

ELISA tests

96 well microtitre plates (Nunc Immuno Plates) were incubated overnight at 4 °C with cyst homogenates (6 µg/ml) of *G. rostochiensis, G. pallida, H. goettingiana, H. schachtii,* or *H. avenae.* Plates were also incubated with 10 µg/ml of actin from bovine muscle, alpha-actinin, myosin, tropomyosin and paramyosin (Sigma). Indirect ELISA test was performed as described previously in Robinson *et al.* (1993). The antibody tested was from tissue culture supernatant of MAb RES GRj-2 and the secondary antibody was a horseradish peroxidase conjugate of goat anti-mouse polyvalent immunoglobulins (1:1000) (Sigma). Negative controls consisted of wells coated with 50 µl of PBS, probed with the same antibody. Assays were performed twice.

Isoelectric focusing (IEF)

Analytical isoelectric focusing was performed with cyst homogenates as described in Robinson *et al.* (1993), on 5 % polyacrylamide gel containing ampholines with a pH range of 3-10 (Pharmacia), using a Multiphor II Electrophoresis Unit (Pharmacia). After focusing, the gels were either stained with Coomassie brilliant blue R250 or electrotransferred to a nitrocellulose membrane to probe with antibodies (Towbin *et al.,* 1979).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted in a vertical slab gel unit (ATTO Corporation) as described by Laemmli (1970), using a 12.5 % (w/v) acrylamide separation gel plus 4 % (w/v) acrylamide stacking gel and 10 µg of protein per lane. Electrophoresis of cyst homogenates was also done with Pharmacia precast minigels (PhastGel 12.5 % homogeneous medium), which requires less biological material, using a Pharmacia LKB PhastSystem for protein separation according to manufacturer's instructions.

Western blotting

Proteins from SDS-PAGE and IEF gels were transferred onto nitrocellulose membrane (NCP, Schleicher & Schuell) in transfer buffer (39 mM Glycine, 48 mM Tris, 0.037 % SDS, 10 % methanol) using a Multiphor II NovaBlot Electrophoresis Transfer Unit (Pharmacia) (Towbin *et al.,* 1979). The NCP containing the molecular weight markers (or pI markers for IEF gels) was cut and stained with 0.1 % amido black, and the remaining NCP containing the transferred proteins was subjected to immunoblotting as described in Robinson *et al.* (1993). Western blots of *G. rostochiensis* and *G. pallida* cyst homogenates along with bovine muscle actin and actinin from chicken gizzard smooth muscle, were probed with MAb RES GRj-2 or rabbit polyclonal antiserum raised to purified actin from chicken back muscle (Sigma).

Alternatively, immunodetection was by enhanced chemiluminescence reagent, a more sensitive reagent for horseradish peroxidase to develop Western blots from minigels (ECL Western blotting detection system, from Amersham International plc). The blot was immersed in ECL Western blotting solution for 1 min and exposed to Hyperfilm-ECL (Amersham) for another 1 min. The film was then developed and photographed immediately.

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The specific antigen recognised by MAb RES GRj-2 was localised by immunofluorescence in cryostat sections of J2 and 1 month-old gravid white females of *G. rostochiensis* and *G. pallida*. A frozen block of nematode pellet in Tissue-teck OCT compound (Miles laboratories) was prepared by rapid freezing in liquid nitrogen. Sections 5-6 µm thick were collected in multispot slides (Agar Scientific), air dried for at least 2 h and fixed in cold acetone for 10 min. The sections were air-dried for 10 min and stored at – 20 °C until required.

The pre-fixed cryostat sections were soaked in 0.2 % Triton X-100 in PBS for 30 min, rinsed briefly in PBS, and then incubated with goat serum 1/50 dilution in 0.01 M PBS pH 7.2 for 20 min. The sections were then incubated overnight at 4 °C with tissue culture supernatant of MAb RES GRj-2. The sections were rinsed three times with 0.01 M PBS pH 7.2 and incubated for 45 min at room temperature with goat anti-mouse IgM fluorescein isothiocyanate conjugate (FITC) diluted 1/50 in 0.01 M PBS pH 7.2. After rinsing the sections with PBS, they were mounted in Citifluor (Agar Scientific) and examined using an Olympus BH-2 microscope fitted with an epi-fluorescence attachment. Negative controls consisted of sections treated in the same way but with omission of the primary antibody, omission of the secondary antibody, or use of an irrelevant MAb with the secondary antibody and no fluorescence was observed.

Results

MAbs were produced against crude homogenates of J2 of *G. rostochiensis* and 336 cell lines were screened by ELISA for reactivity with homogenates of the two species of PCN. MAb RES GRj-2 was cloned by limiting dilution and determined to be of isotype IgM. It showed a slight recognition of *G. pallida* and bound more strongly to *G. rostochiensis,* the same occured when 1 % DOC was incorporated in the extraction buffer, but the antigen was extracted more efficiently (Table 1). Cross-reactivity of the antibody was observed with homogenates from other cyst forming nematodes such as *H. goettingiana* and *H. schachtii* but not *H. avenae*.

Table 1. *Binding of MAb RES GRj-2 to nematode homogenates.*

Nematode species	ELISA reaction (OD 450)*
G. pallida	0.093 ± 0.001
G. rostochiensis	0.407 ± 0.01
G. pallida + DOC**	0.515 ± 0.002
G. rostochiensis + DOC**	1.282 ± 0.03
H. goettingiana	0.118 ± 0.001
H. schachtii	0.312 ± 0.04
H. avenae	0.020 ± 0.001
Control	0.018 ± 0.002

* Values are absorbance means ± standard deviation at 405 nm;
** DOC = sodium deoxycholate.

Cryosections of J2 and gravid white females were used in immunofluorescence studies with MAb RES GRj-2. Autofluorescence at wavelengths close to FITC was observed in the cephalic framework and stylet, a phenomenon which occurs commonly in plant parasitic nematodes and could readily be distinguished by colour from specific FITC fluorescence. A photograph of a longitudinal cryostat section of *G. rostochiensis* J2 probed with MAb RES GRj-2 shows that it reacts with the layer of somatic muscle located beneath the epidermis (Fig. 1A). A cross-section of a *G. pallida* J2 shows the bright fluorescence arranged in four sectors, matching the meromyarian muscle arrangement of the nematode (Fig. 1B). The antibody did not stain any structures in the females and there was no fluorescence in the negative controls (data not shown).

Since cytoskeletal proteins are well conserved in eukaryotes, we probed several cytoskeletal proteins with MAb RES GRj-2 in an indirect ELISA. The antibody reacted mildly with bovine muscle actin but did not react with alpha-actinin, myosin, paramyosin, or tropomyosin (Table 2).

The MAb and the anti-actin antiserum reacted with a major protein of 42 kDa in *G. rostochiensis, G. pallida,* and bovine muscle actin (Figs 2, 3). Weak binding of the MAb to high molecular weight proteins was also observed in *G. pallida* and *G. rostochiensis*. The isoelectric point varied between the two species of PCN; actin and the major band in *G. rostochiensis* had a pI of 5.72, and in *G. pallida* the single protein band had a pI of

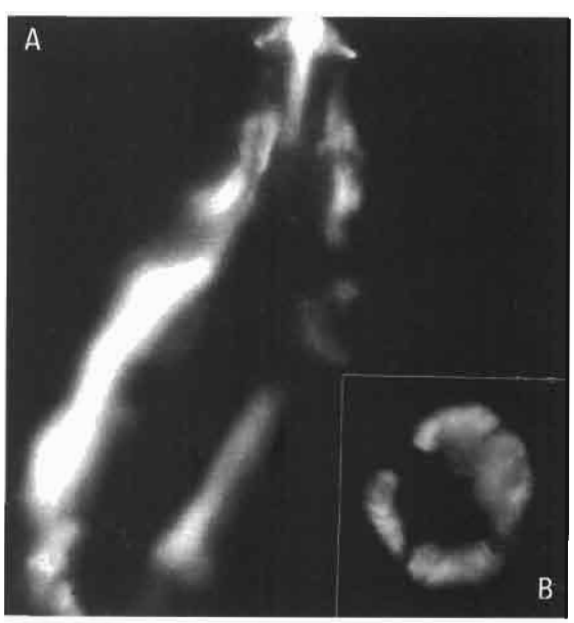

Fig. 1. *Indirect immunofluorescence micrographs of cryostat sections of J2 of* Globodera rostochiensis *and* G. pallida. *A : Longitudinal section of J2 of* G. rostochiensis; *B : Cross-section of J2 of* G. pallida.

Table 2. *Reactivity of MAb RES GRj-2 with cytoskeletal proteins.*

Protein	ELISA reaction (OD 450)*
Actin (bovine muscle)	0.152 ± 0.01
Alpha-actinin	0.029 ± 0.002
Myosin	0.030 ± 0.03
Paramyosin	0.027 ± 0.001
Tropomyosin	0.029 ± 0.01
Control	0.023 ± 0.005

* Values are absorbance means ± standard deviation at 405 nm.

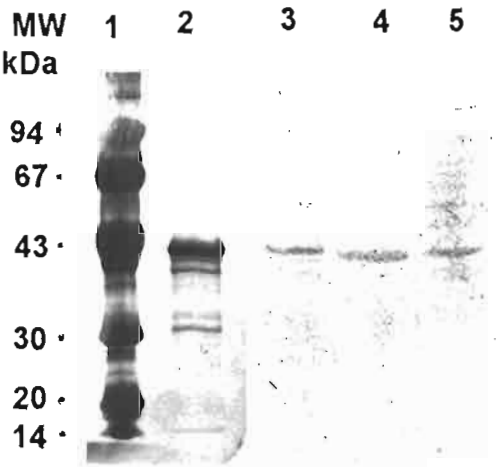

Fig. 2. *Silver staining of SDS-PAGE and Western blot probed with MAb RES GRj-2. A : PhastGel 12.5 % SDS-PAGE stained with silver (lane 1 contains the molecular weight markers and lane 2 actin from bovine muscle); B : Western blot of a 12.5 % SDS-PAGE PhastGel probed with the MAb RES GRj-2 (lane 3 :* Globodera rostochiensis *cyst homogenate; lane 4 : bovine muscle actin; lane 5 :* G. pallida *cyst homogenate).*

5.46 (Fig. 4). Additional weak binding of the antibody to *G. rostochiensis* and actin proteins of different pi was also seen.

Discussion

In immunofluorescence assays the binding of antibody RES GRj-2 was restricted to somatic muscle of J2 and no binding was observed to gravid white females. The females are sedentary and body wall muscles are limited and somewhat atrophied (Shepherd & Clark, 1978). However, the antigen was detected by ELISA and immunobloting in homogenates prepared from cysts, possibly indicating that it was extracted from muscle cells of unhatched J2. However, we cannot discard the possibility of the antigen being present in cytoplasmic filaments in females and not being detected by the immunofluorescence assay. MAbs reacting with somatic

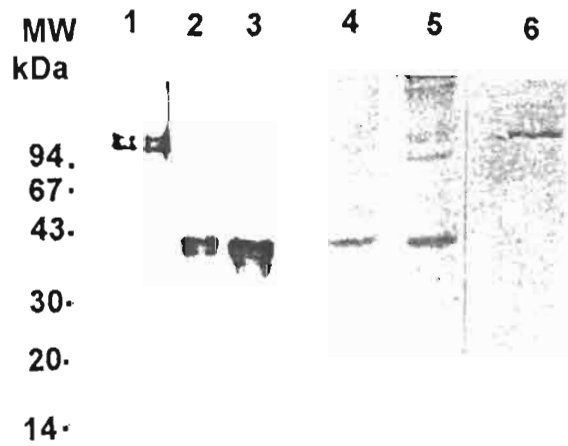

Fig. 3. *Western blot of 12.5 % SDS-PAGE probed with anti-actin polyclonal antibody. Lane 1 : alpha-actinin; Lane 2 : bovine muscle actin; Lane 3 : actin from rabbit heart; Lane 4 :* Globodera rostochiensis *cyst homogenate; Lane 5 :* G. pallida *cyst homogenate; Lane 6 : alpha-actinin.*

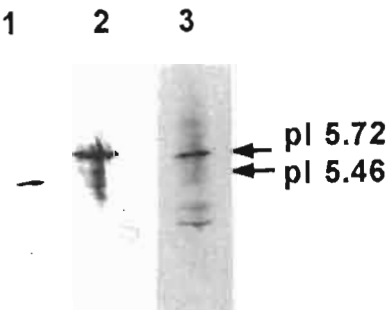

Fig. 4. *Immunoblot of an IEF gel. Lane 1 :* Globodera pallida *cyst homogenate; Lane 2 :* G. rostochiensis *cyst homogenate; Lane 3 : bovine muscle actin.*

muscle of J2 of *Meloidogyne* spp. have been shown to bind to granules of the dorsal oesophageal gland of females (Davis *et al.*, 1992, 1994). However, the same phenomenon was not observed with MAb RES GRj-2, perhaps indicating that the actin recognised by this antibody is only present in muscle cells of J2 and is not involved in granule transport. It has also been postulated by Boer *et al.* (1992), from analysis of two-dimensional electrophoresis gels, that *G. rostochiensis* may have three isoelectric point variants of actin. They also suggested that two of these isoforms were restricted to the body-wall muscle cell since they were absent from females. The third actin isoform is present in all stages and it was postulated that this originated from muscle and non-muscle cells and that the actin present in sedentary females may originate from cytoplasmic actin filaments.

The protein recognised by the MAb seems to have similarity with mammalian muscle actin since they share some common properties, such as molecular size and

isoelectric point. Cross-reactivity of the MAb with bovine muscle actin reinforces the suggestion that the antibody recognises a muscle protein related to actin. The MAb may be reacting with a highly conserved antigenic determinant since binding occurred with actins from a range of widely different species, e.g., chicken, cattle, etc. The MAb also reacted with other cyst nematodes such as *Heterodera schachtii* and *H. goettingiana,* which had, respectively, a weak and a strong cross-reactivity with MAb RES GRj-2 in ELISA assays. However, in *G. pallida* the antibody affinity was consistently lower than in *G. rostochiensis,* indicating a difference in antigenicity of the protein in the two species. The MAb was raised to *G. rostochiensis* and, although it recognises a common epitope in *G. rostochiensis* and *G. pallida,* the different isoforms of the protein, as indicated by IEF, may affect the affinity of MAb binding between the two species of PCN. The MAb had additional weak reaction showing high molecular weight bands in *G. pallida* and *G. rostochiensis,* and actin separated by IEF, which perhaps indicates cross-reactivity of the antibody with multiple isoforms of actin or binding to proteolytic cleavage products retaining anti-actin activity. Proteins exibiting intraspecific and interspecific variation due to difference in their electrical charge, so called isoelectric point variant proteins, have been shown before to occur in PCN by two-dimensional electrophoresis (Bakker, 1987) and by probing with MAbs (Robinson *et al.,* 1993; Curtis & Evans, 1994). This may be due to amino acid substitutions at the molecular level, which seems to account for some of the variation in the protein profiles of the two species of PCN.

Increased binding of the MAb to PCN homogenates was observed in ELISA using nematode antigen extracted with detergent. A similar phenomenon was observed by Yasuda *et al.* (1988), who were able to extract more actin from detergent-treated parasites than from intact ones. In order to exert a mechanical or contractile force, actin filaments are often anchored in cell membranes (Alberts *et al.,* 1983). Therefore, homogenisation of the nematodes with detergent, which causes extraction of cytoplasmic membranes together with other soluble components, may have helped to expose or extract actin bound to nematode membranes.

Actin is the most abundant protein and it has been well conserved during evolution; actins from highly, divergent sources still share amino acid identities in excess of 70 % (Schwob & Martin, 1992). Nevertheless, significant differences are found in actin with the existence of multiple forms and species-specific antibodies reported to certain actins (Groschel-Stewart *et al.,* 1977; Sympson *et al.,* 1984; Pahlic, 1985). In *Naegleria,* a difference in the actin sequence has been shown, perhaps due to the absence of N'-methylhistidine (Fulton *et al.,* 1986). This paper reports the identification of different isoforms and immunolocalisation of a putative actin in the two species of PCN, using a monoclonal antibody. It also shows that the use of nematode cryosections is an excellent method for immunolocalisation of nematode structures with MAbs, because of mild fixation method employed allows preservation of most antigens.

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