THE YOLK POLYPEPTIDES OF A FREE-LIVING RHABDITID NEMATODE

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Abstract—1. The yolk proteins of hermaphrodite Dolichorhabditis sp. (Nematoda, Rhabditida) are composed of at least three polypeptides: VT1, VT2 and VT3 with molecular masses of 175.2, 107 and 82 kDa respectively.
2. All three yolk polypeptides make up at least one native protein complex which can be resolved by PAGE.
3. The yolk proteins are glycosylated and can be isolated by chromatography in Con A-Sepharose.
4. Partial chymotryptic hydrolysis shows that VT2 is different from its C. elegans homologue, YP115.
5. The main polypeptides synthesized by whole animals are the yolk components which are actively secreted in the incubation medium.

INTRODUCTION

The accumulation of reserves in the growing oocytes of oviparous animals has been termed vitellogenesis (Wahl et al., 1981). One characteristic of this phenomenon is the uptake by the oocytes of a huge amount of protein synthesized in extraovarian sites (Wallace, 1985). These accumulated proteins belong to a class of lipoproteins known as vitellogenins (Wahl, 1988). The vitellogenin synthesis is frequently restricted to the females of the species at a given time in their development or physiological stage (Wahl, 1988).

Nematodes have been shown to synthesize a group of yolk protein precursors in their intestinal cells (Kimble and Sharrock, 1983). In Caenorhabditis elegans the vitellogenins consist of four polypeptides (YP170A, \( M_r = 188 \) kDa; YP170B, \( M_r = 186 \) kDa; YP115, \( M_r = 109 \) kDa, and YP88, \( M_r = 83 \) kDa) which are arranged in two complexes exhibiting different sedimentation velocities (Sharrock et al., 1990). Although the genomic organization and complete amino acid sequence of all these yolk polypeptides are known (Heine and Blumenthal, 1986; Spieth et al., 1991), a thorough comparative analysis of other nematode species is necessary to gain a better understanding of how they evolved in this group of animals (Byrne et al., 1989).

Here we describe for the first time the characterization of the yolk polypeptides of a free-living nematode different from C. elegans but belonging to the same family. This species was isolated by us at S. Paulo, Brazil and has been provisionally put in the genus Dolichorhabditis (L. Carta, personal communication, 1991). The population we cultivated in the laboratory (strain B6/D6) was composed exclusively of hermaphrodite animals which makes easier the isolation and study of yolk proteins. Recent results have shown that this species is sufficiently distant from C. elegans to be distinguished at the DNA level (Winter and Blumenthal, unpublished results) although they are morphologically very similar.

MATERIALS AND METHODS

Animals

Dolichorhabditis sp. hermaphrodites were initially isolated from the soil at São Paulo and subsequently grown in a monoxenic medium basically as described by Brenner (1974). Isogenic strains were obtained by starting the cultures with single individuals. The strain used in this work is termed B6/D6. The adult size of this strain is approximately 1.0 mm long with 12 chromosomes in the diploid cells (Dergovics and Winter, in preparation). Caenorhabditis elegans, Bristol strain (N2), was given by Dr. Thomas Blumenthal from a stock maintained at the Department of Biology, Indiana University, Bloomington, IN.

Maintenance of stocks

Both C. elegans (strain N2) and Dolichorhabditis sp. (strain B6/D6) were grown in solid support (NGM Agar: Brenner, 1974) with a lawn of E. coli strains NA 22 (for C. elegans) or MI 178 (CGSC-5680; carA, relA, metB, thr, Hfr) (for Dolichorhabditis sp). The animals were transferred with a sterile platinum wire from one plate to the other. Dolichorhabditis sp. does not form dauer larvae and if left to themselves the plates have no living worms 10 days after the bacteria are exhausted.

Mass liquid culture

The techniques developed for C. elegans (Sulston and Brenner, 1974) were used with some modifications for Dolichorhabditis sp. and C. elegans. Instead of agitating the culture in a shaker, aeration was obtained with a small arium compressor.

Abbreviations—VT, vitellin; YP, yolk polypeptide; Con A, concanavalin A; PMSF, phenylmethylsulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; NP-40, nonidet P-40; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; \( M_r \), relative molecular mass; Da, dalton.
Egg isolation

Dolichorhabditis sp. apparently lays its eggs just after fertilization. As a consequence the laid eggs of this species take longer to hatch and accumulate to great numbers in the plates. We first resuspended in S buffer (Brenner, 1974) the contents of plates just before the bacteria were exhausted. Filtering this suspension with a 30 μm nylon net, a large amount of eggs were obtained with very few adults and L1s. The eggs obtained by this procedure were dissolved in sample buffer and analyzed by SDS-PAGE.

Homogenization of animals

The worms were homogenized by two different procedures; one yielded the homogenate used for affinity chromatography purification and the other the homogenate used on PAGE electrophoresis.

(a) For affinity chromatography. The worms grown in a liquid medium as described above were first concentrated under gravity. These worms were then packed by brief centrifugation at low speed. Four grams of packed worms (adults plus larvae and eggs) of Dolichorhabditis sp. strain B6/D6 were resuspended in 30 ml of homogenization buffer A (2 x (1.2 M NaCl; 2 mM CaCl₂; 2 mM MgCl₂; 1 mM PMFS; 2 mM iodoacetamide; 40 mM Tris pH 7.4) containing 1 mM PMFS and 1 mM iodoacetamide. The worms were disrupted by intermittent sonication at 4°C until no intact animal was detected by phase microscopy (approximately 10 min). The homogenate was centrifuged at 750 g for 10 min at 4°C. The supernatant was then centrifuged at 12,000 g for 10 min at 4°C. The supernatant of the second centrifugation (S10) was diluted twice with 1 x homogenization buffer A and immediately applied to a Con A-Sepharose column (Pharmacia Fine Chemicals, Uppsala, Sweden). Homogenization of C. elegans grown in bulk quantities was done exactly as described by Krishna et al. (1988), starting with 5-7 g of a mixed worm population with no dauer larvae.

(b) For PAGE. The homogenization was done as described by Sharrock (1983) with some modifications. Three to four grams of a mixed population of Dolichorhabditis sp. strain B6/D6 were resuspended in homogenization buffer B (5 mM MgCl₂; 5 mM CaCl₂; 250 mM sucrose and 50 mM sucrose and 50 mM Tris pH 8.0). The suspension was then dropped slowly over liquid nitrogen in a porcelain mortar and the frozen pellets were then reduced to a powder. The frozen powder was allowed to melt in an ice bath and then centrifuged for 10 min at 1500 g at 4°C. The precipitate was washed twice with homogenization buffer, resuspended in 10 ml of the same buffer and NP-40 was added to 0.1% (v/v) final concentration. This solution, which was rich in yolk proteins (Sharrock et al., 1990), was then ultracentrifuged for 4 hr at 112,500 g at 4°C. The supernatant, which contained the solubilized yolk proteins was submitted to discontinuous cylinder gel electrophoresis.

Con A-Sepharose affinity chromatography

This was carried out as described by Krishna et al. (1988) with some modifications. All steps were done at 4°C. Approximately 30 ml of worm homogenate was applied to a Con A-Sepharose column with a gel bed of 7.8 ml (1 cm diameter) at 10 ml/hr. After all the homogenate was applied the column was washed overnight at 5 ml/hr with 1 x homogenization buffer until A₂₈₀nm was near zero. The proteins bound to the column were eluted with homogenization buffer containing 100 mM each of 2-methyl mannoside and 2-methyl glucoside. Fractions containing A₂₈₀nm above 0.05 were analyzed by SDS-PAGE.

SDS-PAGE

Protein samples were dissolved in sample buffer and submitted to SDS-PAGE in slabs according to Winter et al. (1985). Protein staining was done with Coomassie Brilliant Blue R (Winter et al., 1977) or by the silver-nitrate method (Merril et al., 1983).

Fig. 1. Micrographs of the anterior portion of Dolichorhabditis sp. and C. elegans. The animals were anesthetized with 0.01% NaN₃, put over a thin 4% agarose pad and covered with a coverslip. The micrographs were made with Nomarski optics. (a) Anterior portion of C. elegans; (b) anterior portion of Dolichorhabditis sp. pc = procorpus; mc = metacorpus; pb = posterior bulb.
Discontinuous gel electrophoresis

This was done in glass tubes according to Davis (1964) using the same solutions used for SDS-PAGE without SDS. The runs were made in 7% gels according to Winter et al. (1985). All samples were run in duplicates. After electrophoresis, the gels were removed from the glass tubes and one of them was frozen at $-20^\circ$C while the other was stained with Amido Black (Panyim and Chalkley, 1969). The frozen gel was sliced into 1.7 mm fractions. The proteins in these fractions were eluted in SDS sample buffer at 100°C and submitted to SDS-PAGE.

Protein molecular mass estimation

The molecular mass was estimated by SDS electrophoresis according to Lambin et al. (1976), utilizing linear polyacrylamide gradients or according to Plikaytis et al. (1986) utilizing gels of single polyacrylamide concentration ($T = 10\%$). The molecular mass markers were: human erythrocyte z-spectrin (240 kDa) and $\beta$-spectrin (220 kDa); myosin heavy chain (200 kDa); $\beta$-galactosidase (166 kDa); phosphorylase b (92 kDa); bovine serum albumin (66 kDa); catalase (60 kDa); egg white albumin (45 kDa); lactate dehydrogenase (36 kDa); carbonic anhydrase (30 kDa); soybean trypsin inhibitor (20 kDa). Except for the human

Fig. 2. Analysis of yolk proteins from eggs of Dolichorhabditis sp. The photograph shows a SDS-PAGE of proteins from Dolichorhabditis sp. eggs and molecular mass markers. The electrophoresis was run in a 10% polyacrylamide gel slab and stained with Coomassie Blue R. The molecular mass curve at the bottom of the figure was obtained using the data shown in the photograph adjusted according to Plikaytis et al. (1986). M = Myosin heavy chain; G = $\beta$-galactosidase; P = phosphorylase b; B = bovine serum albumin; E = egg albumin; C = carbonic anhydrase; T = soybean trypsin inhibitor; HMW = high molecular mass markers; LMW = low molecular mass markers; O = egg proteins.
erythrocyte spectrins (which were given by Dr. Julio Pudles, Department of Parasitology—ICB USP) all other molecular mass markers came from commercial sources (Pharmacia Fine Chemicals and Bio-Rad, Richmond, CA).

**Chymotrypsin A partial digestion**

Yolk proteins from both *C. elegans* and *Dolichorhabditis* sp., purified by Con A–Sepharose chromatography were subjected to SDS-PAGE in a T = 10% gel. The gels were rapidly stained and dried. The appropriate bands were cut and subjected to Chymotrypsin A partial digestion and fractionated in a T = 15% SDS–PAGE as previously described (Winter et al., 1985). This gel was silver-stained as described by Merrill et al. (1983).

$^3$H-Leucine incorporation and fluorography

$^3$H-Leucine incorporation was made as described by Kimble and Sharrock (1983) in a saline solution developed for *C. elegans* (Hirsh et al., 1976). Ten worms were cut at the distal part of the oesophagus and incubated for 1 hr at 20°C in 5 μl of saline containing 25 μCi of L-(4,5-$^3$H)-leucine (145 Ci/mmol). After incubation, the animals were dissolved in SDS sample buffer as described above. This sample was then submitted to SDS–PAGE in a 10% polyacrylamide slab gel.

After electrophoresis the gels were processed for fluorography as described by Bonner and Laskey (1974) and exposed to X-ray film at –70°C.

**Fig. 3.** Molecular mass determination of VT1. Yolk proteins from *Dolichorhabditis* sp. purified by affinity chromatography in Con A–Sepharose were subjected to electrophoresis in SDS-PAGE in a linear gradient of 4–15% T. The distance migrated by the standards (α S = human erythrocyte α-Spectrin; β S = human erythrocyte β-Spectrin; P = phosphorylase b; BSA = bovine serum albumin; C = catalase; O = egg white albumin; LDH = lactate dehydrogenase; CA = carbonic anhydrase) were transformed in %T and plotted as the square root, as described by Lambin et al. (1976). The correlation coefficient of the curve is $r = 0.9984$. VT1 migrated to a T = 7.41%.

**Fig. 4.** Discontinuous gel electrophoresis of yolk proteins from *Dolichorhabditis* sp. Proteins of the particulate fraction of a worm homogenate (see Material and Methods) were submitted to discontinuous electrophoresis in a gel cylinder at a polyacrylamide concentration of 7%. This gel was stained with Amido Black and the result is shown at the top of the figure. An identical cylinder was fractionated and fractions 1–15 (each one with 1.7 mm) were submitted to SDS–PAGE in a 10% gel slab. This gel was silver-stained and the result of this second electrophoresis is shown at the bottom of the figure. At the left of the SDS–PAGE is shown the relative migration rate (${R_m}$) scale. At the right is shown the position of the yolk polypeptides (VT1, VT2 and VT3) and the myosin heavy chain (M). The arrow in both electrophoreses shows the position of the native yolk proteins; T = stacking gel.
RESULTS

Morphology

Adult hermaproditic females of Dolichorhhabditis sp. are almost the same size as C. elegans. The only difference which can be readily seen at the light microscopy level is the oesophagus morphology. The oesophagus of Dolichorhhabditis sp. does not show the conspicuous bulb-like metacorpus which is clearly observed in C. elegans (Fig. 1a,b).

Egg proteins

The yolk proteins Dolichorhhabditis sp. were first identified in eggs. Eggs obtained as described in Material and Methods were dissolved in sample buffer and submitted to SDS-PAGE. The main egg polypeptides observed after staining were labeled VT1, VT2 and VT3 (Fig. 2). The molecular mass of VT2 and VT3 were obtained from a 10% gel and shown to be 107 and 83 kDa respectively (Fig. 2). The molecular mass of VT1, which could not be obtained from this gel, was calculated as 175.2 kDa (Fig. 3) from a gradient gel (not shown). All these values for the Mr of VT1, VT2 and VT3 are approximately the same obtained for KP 170A+B, KP115 and KP88 from C. elegans yolk (Sharrock et al., 1990).

Native yolk polypeptide complexes

Worm homogenate, obtained as described in Material and Methods, was submitted to discontinuous electrophoresis with SDS. After the run the cylinder was fractionated and the polypeptides present in each fraction were analyzed by SDS-PAGE. Figure 4 shows the results obtained. The cylinder electrophoresis show a prominent band approximately 2 cm from the origin. This region contains the three polypeptides which were found in the eggs. This shows that they are part of the same protein or of proteins which have nearly the same electrophoretical behavior. In Fig. 2 VT3 is more stained than the other yolk polypeptides. This is due to its greater affinity for silver salts. The reason for this phenomenon is not known but has already been noticed with other proteins (Merril et al., 1983).

Affinity chromatography of the yolk

After homogenization as described in Material and Methods Dolichorhhabditis sp. proteins were submitted to affinity chromatography in Con- A-Sepharose. Figure 3 shows the results obtained with this procedure. Part of the protein of the homogenate is retained in the column and is eluted with α-methyl

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**Fig. 5.** Purification of Dolichorhhabditis sp. yolk proteins by Con A-Sepharose affinity chromatography. The graphic on the top of the figure shows the absorbancy profile of the proteins eluted from the column with α-methyl mannoside and α-methyl glucoside. The homogenate was applied to the column and washed with 10 volumes of homogenization buffer. The bound proteins were then eluted with α-methyl glucoside and α-methyl mannoside as described in Material and Methods. The graph at the bottom shows the polypeptides present in fractions 3–9. FT corresponds to the proteins present in the flow-through and S10 to those present in the homogenate applied to the column.
mannoside and α-methyl glucoside. This fraction contains, among other polypeptides, those that make up the native yolk proteins (Fig. 5). Moreover, all the yolk polypeptides present in the initial homogenate (Fig. 5, S10) are retained in the column. This can easily be seen when we analyze the polypeptides present in the flow-through (Fig. 5, FT) which is completely devoid of VT1, VT2 and VT3.

**VT2 and YP115 comparison**

The fact that Dolichorhabditis sp. and *C. elegans* are so similar at the morphological level prompted us to analyze the peptide map of VT2 of *Dolichorhabditis* sp. and YP 115 of *C. elegans*. Both YP115 and YP88 are originated by the cleavage of a common 193 kDa precursor (Sharrock et al., 1990). This precursor is coded by the vit-6 gene (Spith and Blumenthal, 1985) which is the only autosomal vitellogenin gene from *C. elegans* (Heine and Blumenthal, 1986) being located at linkage group IV.

Figure 6 shows the results of the partial digestion of VT2 and YP115 by chymotrypsin. Without chymotrypsin treatment the polypeptides show only a smear below them (Fig. 6a,b). When they are partially digested with chymotrypsin we can see that both polypeptides originate a unique digestion pattern (Fig. 6c,d). VT2 shows a prominent band with an Rm of approximately 0.45 which is not present in the YP115 profile. Both polypeptides show very strong bands which migrate near the undigested species (Rm 0.12).

**Yolk polypeptides synthesis**

When animals whose anterior parts were sectioned were incubated in a medium containing 3H-Leu we could see that the main proteins synthesized by the worms were the yolk components (Fig. 7). The analysis of the incubation medium by SDS-PAGE and fluorography shows that VT1 (and probably also the other yolk peptides) is actively secreted, as occurs with *C. elegans* (Kimble and Sharrock, 1983).

**DISCUSSION**

We have shown that the yolk proteins of *Dolichorhabditis* sp. are composed of at least three polypeptides: VT1 (175.2 kDa), VT2 (107 kDa) and VT3 (82 kDa). These yolk polypeptides have molecular masses which are very similar to those calculated for the *C. elegans* yolk polypeptides (Sharrock et al., 1990).

As in *C. elegans* the yolk polypeptides of *Dolichorhabditis* sp. form native multimers. In *Dolichorhabditis* sp. this was shown by two-dimensional electrophoresis. Contrary to what has been shown in *C. elegans* (Sharrock et al., 1990) we did not detect more than one native protein complex with different polypeptide composition. Nevertheless, a faint band can be seen in the disc electrophoresis just before the main yolk protein band (see Fig. 4). This could be the band that corresponds the YP 170A dimer detected in the yolk proteins of *C. elegans* (Sharrock et al., 1990). This last assumption is not confirmed by the second dimension run. The results obtained by Con
The yolk polypeptides of *Dolichorhabditis* sp. are actively synthesized and secreted in the incubation medium by whole adult animals. The synthesis of VT1 accounts for more than 50% of the proteins synthesized by the whole animal (Fig. 7). This is in accordance with data obtained in *C. elegans* which show that the vitellogenin genes transcripts are among the most abundant mRNAs in the whole worm (Blumenthal et al., 1984).

The results presented herein have shown that the yolk proteins of *Dolichorhabditis* sp. and *C. elegans* have many similarities in chemical behavior and structure. However, when compared by partial digestion, differences could be shown among *Dolichorhabditis* sp. VT2 and *C. elegans* YP115. This last result is in accordance with preliminary data obtained on the sequence of one of the vitellogenin genes of *Dolichorhabditis* sp. (Winter and Blumenthal, unpublished results).

Like *C. elegans*, *Dolichorhabditis* sp. can be easily maintained in the laboratory. This characteristic and its morphological and biochemical similarities to *C. elegans* make it a useful organism or which to carry out comparative studies on nematodes.

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