Comparative Biochemistry and Physiology, Part B 151 (2008) 330–335

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Vitellin-binding proteins in the nematode *Oscheius tipulae* (Nematoda, Rhabditida)

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ARTICLE INFO

Article history:
Received 16 June 2008
Received in revised form 24 July 2008
Accepted 29 July 2008
Available online 3 August 2008

Keywords:
Caenorhabditis elegans
Ligand blotting
Nematode
Oscheius tipulae
Vitellin
Vitellogenin receptor

ABSTRACT

We describe the first application of a non-radioactive ligand-blotting technique to the characterization of proteins interacting with nematode vitellins. Chromatographically purified vitellins from the free-living nematode *Oscheius tipulae* were labeled with fluorescein *in vitro*. Ligand-blotting assays with horseradish peroxidase-conjugated anti-fluorescein antibodies showed that labeled vitellins reacted specifically with a polypeptide of approximately 100 kDa, which we named P100. This polypeptide is a specific worm's vitellin-binding protein that is present only in adult worms. Blots containing purified *O. tipulae* vitellin preparations showed no detectable signal in the 100 kDa region, ruling out any possibility of yolk polypeptides self-assembling under the conditions used in our assay. Experiments done in the presence of α-methyl mannoside ruled out the possibility of vitellins binding to P100 through mannose residues. Triton X-114 fractionation of whole worm extracts showed that P100 is either a membrane protein or has highly hydrophobic regions.

1. Introduction

Nematodes and other species depend on the ability to produce eggs at high rates to colonize new habitats. The intensive egg production during adult life represents, for free-living species, an adaptation to short life cycles, and for parasitic species, effective host colonization. A single fertilized female of the human parasite *Ascaris lumbricoides* produces around 300,000 eggs daily for several years in the same host (Sinniah, 1982). Thus, it is important to unravel the mechanisms that are involved in oogenesis to understand the different life cycles of the animals in this phylum.

Yolk proteins, also known as vitellins, are the major proteins of nematode egg yolk and originate from a dimeric lipoprotein called vitellogenin (VTG) (Winter, 2002), which is synthesized outside the ovary and taken up by the growing oocytes through receptor-mediated endocytosis. Inside the nematode oocyte, the vitellogenin is processed without proteolytic cleavage and stored in yolk granules (probably modified endosomes) until it is used by the developing embryo or by the recently emerged larvae (Grant and Sato, 2006). Vitellogenins are ancient members of the large lipid transfer protein (LLTP) super family, which also includes human apolipoprotein B (apoB), microsomal triglyceride proteins (MTP) and insect apolipophorins (Babin et al., 1999). Although those proteins perform rather distinct functions, they are structurally and phylogenetically related (Mann et al., 1999). Phylogenetic analysis of an alignment of the first 650 amino acids of several lipoproteins showed that apoB, MTP and insect lipophorins share a common ancestry with the VTGs of nematodes (Babin et al., 1999).

To date, most of the work on nematode vitellogenesis has been done in the classical biological model *Caenorhabditis elegans*, a small free-living nematode member of the family Rhabditidae. Vitellogenin biosynthesis in *C. elegans* occurs inside the intestinal cells (Kimble and Sharrock, 1983), and the yolk protein precursors are secreted into the body cavity before being taken up by the growing oocytes. There are four polypeptides in the yolk, YP170A, YP170B, YP115 and YP88 (Sharrock, 1983), and six different genes coding for those polypeptides (Winter, 2002).

Grant and Hirsh (1999) have shown that in *C. elegans* vitellogenin enters the growing oocyte through receptor-mediated endocytosis. Using transgenic lineages expressing GFP (green fluorescent protein)-tagged YP170B, they were able to isolate several mutants with abnormal VTG uptake (Grant and Sato, 2006). One of those mutants lead to the identification of the VTG receptor (VTGR) gene (*rme*-2) (Grant and Sato, 2006), which has a calculated molecular mass of 106 kDa, almost the same size as the vertebrate VTGRs (95–115 kDa) and smaller than the insect VTGRs (180–214 kDa) (Sappington and Raikhel, 1995). The VTGRs, present in the oocyte plasma membrane, are members of the low density lipoprotein-receptor (LDLR) family (Bujo et al., 1994), even though they have functional differences (Shankaran et al., 2007). Some of the VTGRs have been studied in other invertebrates, including arthropods (Roehrkasten and Ferenz, 1986; Roehrkasten et al., 1989; Schonbaum et al., 1995; Sappington et al., 1995; Cho and Raikhel, 2001) and annelids (Hafer et al., 1992).

Experiments designed to examine the interaction between lipoproteins and their receptors were initially performed in solutions

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doi:10.1016/j.cbpb.2008.07.017

containing non-ionic detergents. Since the work of Daniel et al. (1983), the ligand-blotting technique has been successfully applied to several classes of lipoproteins and their cognate receptors, including vitellogenin and VTGR (Stifani et al., 1990). Detection of the lipoprotein-receptor complex in a blot has been achieved through several distinct protocols, including staining with colloidal gold (Roach et al., 1987).

Oscheius tipulae, a rhabditid nematode related to *C. elegans*, is also used as a model for vitellogenesis studies (Winter, 2002; Félix, 2006). The first strain (CEW1) used for those studies was isolated from soil samples collected in the city of São Paulo, Brazil (Winter, 1992). Vitellins have been characterized in CEW1 and are composed of three polypeptides, VT1, VT2 and VT3, of approximately the same size as those of *C. elegans* (Winter, 1992). The gene coding for VT2 and VT3 has been cloned and sequenced; some striking differences from its homologue in *C. elegans* were found (Winter et al., 1996).

We show in this study that there is an interaction between at least one polypeptide present in worm extracts and native vitellins of *O. tipulae* (strain CEW1). We detected binding of vitellins to a 100 kDa protein band *in vitro* with purified vitellin preparations. Cell fractionation demonstrated that the 100 kDa protein band is either membrane localized or has a highly hydrophobic nature.

2. Materials and methods

2.1. Nematode strains and culture conditions

Monoxenic cultures of *C. elegans* (strain Bristol-N2) and *O. tipulae* (strain CEW1) were kept in NGM Agar seeded with *Escherichia coli* NA22 as described by Wood (1988). Mass cultures were grown under vigorous aeration as described by Sulston and Brenner (1974).

2.2. Vitellin purification

All procedures were done in the presence of protease inhibitors (1 mmol/L Pefabloc [4- (2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride] Roche™; 20 μmol/L pepstatin and 20 μmol/L E-64). Worms obtained from asynchronous mass cultures using *E. coli* strain NA22 as a food source were decanted, washed with S buffer (50 mmol/L potassium phosphate, 100 mmol/L NaCl, pH 6.0) and floated over 30% (*w*/*v*) sucrose as described by Sulston and Hodgkin (1988). The floating worms were washed two times in ultra-pure water. The final worm slurry was slowly dropped into liquid nitrogen and stored at −70 °C or used immediately. Frozen worm drops were then reduced to a powder under liquid nitrogen in a porcelain mortar using a porcelain pestle. The powder was defrosted in homogenization buffer (20 mmol/L Tris pH 7.4; 0.6 mol/L sodium chloride; 1 mmol/L $CaCl_2$; 1 mmol/L $MnCl_2$), and the resulting homogenate was centrifuged at 1500 *g* for 5 min. The supernatant was centrifuged again at 10,000 *g* for 5 min, and the supernatant of this spin was used for vitellin purification. Vitellin was purified using a Con A Sepharose column (GE Healthcare) as described by Winter (1992). After Con A affinity chromatography, aliquots from each fraction were submitted to SDS-PAGE analysis. Fractions enriched in the amount of vitellin polypeptides VT1, VT2 and VT3 (Winter, 1992) were pooled and subjected to ion exchange chromatography using a Resource Q column (GE Healthcare) previously equilibrated with buffer A (10 mmol/L Tris pH 8.0; 1 mmol/L EDTA). The adsorbed proteins were eluted from the column with a NaCl gradient from 0.1 to 1.0 mol/L in the same buffer A at a flow of 2.0 mL/min. $A_{280\ nm}$ was continuously recorded, and fractions containing the vitellin polypeptides (as detected by SDS-PAGE) were pooled and dialyzed against PBS (4 mmol/L Na_2HPO_4; 150 mmol/L NaCl). The resulting solution was concentrated inside a dialysis bag using Sephadex G-75 resin (GE Healthcare). Purified vitellins were kept at 4 °C in the presence of protease inhibitors and used within 1 week after purification.

2.3. Protein labeling with fluorescein

Purified vitellin (VT) or bovine serum albumin (BSA) was dissolved in 0.1 mol/L carbonate–bicarbonate buffer pH 8.3 and reacted with a 2.5× molar excess [assuming a molecular mass of approximately 400 kDa for the native vitellins (Sharrock et al., 1990)] of Fluorescein-5-EX-succinimidyl ester (Invitrogen/Molecular Probes; cat. nr. F-6130) for 1 h in the dark at room temperature. Afterwards, ammonium chloride was added to a final concentration of 50 mmol/L, and the reaction was incubated for 30 min at 4 °C in the dark. The final mixture was then applied to a Nick column (GE Healthcare) with a Sephadex G-50 gel bed previously equilibrated with 0.1 mol/L carbonate–bicarbonate buffer pH 8.3. The labeled vitellin (VT_f) or BSA (BSA_f) eluted in the void volume (V_0) of the column and was visually monitored with a hand held UV lamp (long wavelength). Fluorescein-labeled proteins were mixed with ultra-pure molecular biology grade glycerol (1:1 v/v) and stored in the dark at −20 °C.

2.4. Membrane proteins partitioning with Triton X-114

Worms were homogenized using a "Mini-Bomb Cell disruption chamber" (Kimble/Kontes, Vineland, NJ, USA) under a nitrogen pressure of 80 kg/cm^2 for 10 min at 25 °C in TBS (25 mmol/L Tris pH 8.0; 137 mmol/L sodium chloride; 2.7 mmol/L potassium chloride) in the presence of protease inhibitors (1 mmol/L Pefabloc [4- (2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride] Roche™; 20 μmol/L pepstatin and 20 μmol/L E-64). The resulting homogenate was successively centrifuged as shown in Fig. 5. Pellets from the centrifugation steps at 600 *g* and 10,000 *g* were resuspended in TBS 1× with 2% precondensed Triton X-114 (v/v) as described by Pryde and Phillips (1986), incubated for 15 min at ice-cold temperature and centrifuged at 10,000 *g* for 10 min at 4 °C. Upper and lower phases were collected. The lower fractions were stored for further analysis, and the upper ones were kept at 37 °C until the solution became opalescent. These samples were subjected to another centrifugation step at 10,000 *g* for 10 min at 4 °C. After this last centrifugation, the upper phase (detergent-depleted) contained soluble proteins and the lower phase (detergent-enriched) contained proteins anchored by GPI structures or by transmembrane domains. All the fractions obtained during this partitioning were precipitated with TCA (trichloroacetic acid) 10% (v/v), washed with acetone 100% and stored dry at −20 °C prior to SDS-PAGE.

2.5. Ligand-blotting assay

Whole worms were directly dissolved in sample buffer [62.5 mmol/L Tris pH 6.8; 2,5% (v/v) β-mercaptoethanol; 2.6% (v/v) SDS; 12.6% (v/v) glycerol; 0.5 mmol/L EDTA; 0.5 mmol/L EGTA; 0.01% (w/v) pyronin Y] by boiling at 100 °C for 10 min. They were then subjected to SDS-PAGE (T=10%), and the proteins were electrophoretically transferred to a nitrocellulose membrane (Hybond-C™, Amershan Biosciences) at 90 mA, for 16 h at 4 °C using a Mini Protean II apparatus (Bio-Rad) containing the transfer buffer [20% (v/v) methanol; 195 mmol/L glycine; 25 mmol/L Tris pH 8.3]. Membranes were stained with Ponceau S and destained with TBS (25 mmol/L Tris pH 8.0; 137 mmol/L NaCl; 2.7 mmol/L KCl). The blot was incubated at room temperature for 2 h with TBS containing 5% of skimmed milk (TBS-milk). After buffer removal, the reaction with VT_f (or BSA_f) occurred at 4 °C in the dark with 0.9 ng of labeled protein mixed with TBS-milk. The following steps were done at room temperature. The blot was washed three times (10 min each) in the dark with TBS and incubated for 2 h with TBS-milk containing anti-fluorescein peroxidase-conjugated antibody (GE Healthcare) diluted 1:1000 (v/v). After another three TBS-washes, the blot was reacted with ECL Plus™ Western Blotting Detection System (GE Healthcare) and exposed to an X-ray film for 4 h at 4 °C.