Comparative Biochemistry and Physiology, Part B 155 (2010) 256–265

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

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

Vitellogenesis in *Bufo arenarum*: Identification, characterization and immunolocalization of high molecular mass lipovitellin during oogenesis

Emma D. O'Brien [a], Ana M. Salicioni [b], Marcelo O. Cabada [a], Silvia E. Arranz [a,*]

[a] Instituto de Biología Molecular y Celular de Rosario (CONICET-UNR) and Área Biología, Facultad de Ciencias Bioquímicas y Farmacéuticas, UNR, Argentina
[b] Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, MA, USA

ARTICLE INFO

Article history:
Received 6 August 2009
Received in revised form 12 November 2009
Accepted 14 November 2009
Available online 20 November 2009

Keywords:
Vitellogenesis
Oogenesis
Lipovitellin
Yolk platelets
Amphibian

ABSTRACT

Vitellogenin (Vtg), a large lipoglycophosphoprotein, is the most important precursor of the yolk proteins, and the major source of nutrients for the developing embryo in oviparous species. After its uptake by the oocytes, Vtg is converted into lipovitellins (high and light) and phosvitin, which are deposited into crystalline yolk platelets. We describe here the presence of two high molecular mass lipovitellin isoforms in *Bufo arenarum* mature oocytes with masses of 113 and 100 kDa, respectively. The amino acid sequence analysis of p113 and p100 peptides showed a high sequence homology between both polypeptides and the complete reported sequences of *Xenopus laevis* vitellogenin. Using specific antibodies, we determined that the Vtg uptake begins early during oogenesis, at the previtellogenic stage, and continues until oocytes have reached their mature status. In addition, we found that large endocytic vesicles mediate Vtg uptake in stage I oocytes, and that the size of the endocytic vesicles declines with oogenesis progression. In terms of the Vtg protein trafficking, we detected the Vtg precursor (190 kDa) in the liver of estradiol-injected females. Finally, we propose a subclassification of *B. arenarum* stage II oocytes into three physiologically and morphologically distinct periods (early, mid and late).

1. Introduction

In egg-laying species, the developing embryo is dependent on the egg components for its nutritional requirements. The major source of nutrients for the developing embryo is the egg yolk. In amphibians, vitellogenin (Vtg), a large lipoglycophosphoprotein, is the most important precursor of the yolk proteins. Vtg is synthesized in the liver in response to estrogen, transported via the bloodstream to the ovary and internalized by the growing oocytes through receptor-mediated endocytosis (Tata and Smith, 1979; Wallace et al., 1973) After its uptake by the oocyte, Vtg is converted into lipovitellins (a high molecular mass lipovitellin –LvH– and a light lipovitellin –LvL–) and phosvitins, which are deposited into crystalline yolk platelets (Opresko et al., 1980). *Xenopus laevis* (Mesobatrachia) has been a suitable model for studies on vitellogenesis (Wallace, 1970; Wallace and Ho, 1972; Yoshitome et al., 2003). Although the existence of two families (A and B) and four subtypes (A1, A2, B1 and B2) of vitellogenin has been shown in *Xenopus* sp., the complete sequence has been determined only for vitellogenins A2 (Gerber-Huber et al., 1987; Wahli et al., 1982; Wallace et al., 1990) and B1 (Yoshitome et al., 2003). *X. laevis* LvH is derived from the amino-terminal of its precursor and has an apparent molecular mass of 115 kDa (Molla et al., 1983). Using higher-resolution analytical procedures, three apo-LvH proteins with molecular masses of 121, 116, and 111 kDa have been characterized *in X. laevis* (Wiley and Wallace, 1981). In species closely related to *Bufo arenarum*, like *Odontophrynus americanus* (Neobatrachia), two isoforms, LvHα and β, with molecular masses of 104.6 kDa and 92.6 kDa, respectively, have been also identified (Winter et al., 1985). Several studies have reported on the mechanism of the Vtg internalization in amphibians (Wall and Patel, 1987; Ward, 1978). However, there is scarce information on Vtg protein processing during the oogenesis in these species. It is known that the growth rate of oocytes is closely related to the rate of the vitellogenin uptake. The fastest rate of growth in *Xenopus* sp. oocytes occurs from mid-stage IV (approximately 0.8 mm diameter) until mid-stage V (1.2 mm diameter), which corresponds to the period of the most pronounced vitellogenin uptake. In the final stages of the oogenesis, the amphibian oocytes acquire an animal–vegetal polarity, showing pigment granules in the animal pole and the yolks platelets localized in the vegetal hemisphere (Danilchik and Gerhart, 1987). *B. arenarum* (*Chaunus arenarum*, Frost et al., 2006) is a common toad (Anura: Bufonidae) in the South American region and has a yearly reproductive cycle. Its ovulation occurs during the spring season. During natural hibernation large populations of uniform sized hormone-responsive follicles exist in arrested meiosis within the ovary. In the present work, two polypeptides, p113 and p110, were

* Corresponding author. Tel./fax: +54 341 4804601.
E-mail address: arranz@ibr.gov.ar (S.E. Arranz).

1096-4959/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpb.2009.11.008

identified as two different isoforms of LvH by MS/MS analysis in *B. arenarum* oocytes. Our work focuses on their biochemical characterization and localization during the oogenesis, and demonstrates that the Vtg uptake begins early during the oogenesis and continues until the oocyte reaches its full, mature size.

2. Materials and methods

2.1. Experimental animals

Sexually mature *B. arenarum* specimens were collected in the neighborhoods of Rosario City and kept in a moist chamber at 12 °C until used. Experiments were performed in accordance with the guide for the care and use of laboratory animals of Facultad de Ciencias Bioquimicas y Farmacéuticas, Universidad Nacional de Rosario.

2.2. Preparation of protein extracts from B. arenarum oocytes

Female specimens were kept in a moist chamber at 20–22 °C for 1 day before stimulation, which was done by intracoelomical injection of a homologous pituitary extract of sexually mature animals. After 10–12 h, oocyte strings were collected from ovisacs (Valz-Gianinet et al., 1991). Degelling was then performed as previously reported (Barisone et al., 2002). Oocytes were washed with 10% v/v Ringer–Tris buffer, homogenized with a Potter-Elvehjem homogenizer, and the vitelline envelopes were separated by filtering the protein extract through a double sheet of a 30-mesh screen. In order to improve the yolk protein recovery, ovulated oocytes were solubilized in a variety of high-ionic strength buffers. Once treated, the samples were centrifuged and supernatants were analyzed by SDS-PAGE (data not shown) to determine the presence or not of the Vtg-related bands. We found that yolk proteins solubility was highest in 6 M guanidine + 5% w/v CHAPS, 6 M guanidine + 50 mM DTT, 2% w/v SDS, or 8 M urea + 2% w/v CHAPS + 50 mM DTT.

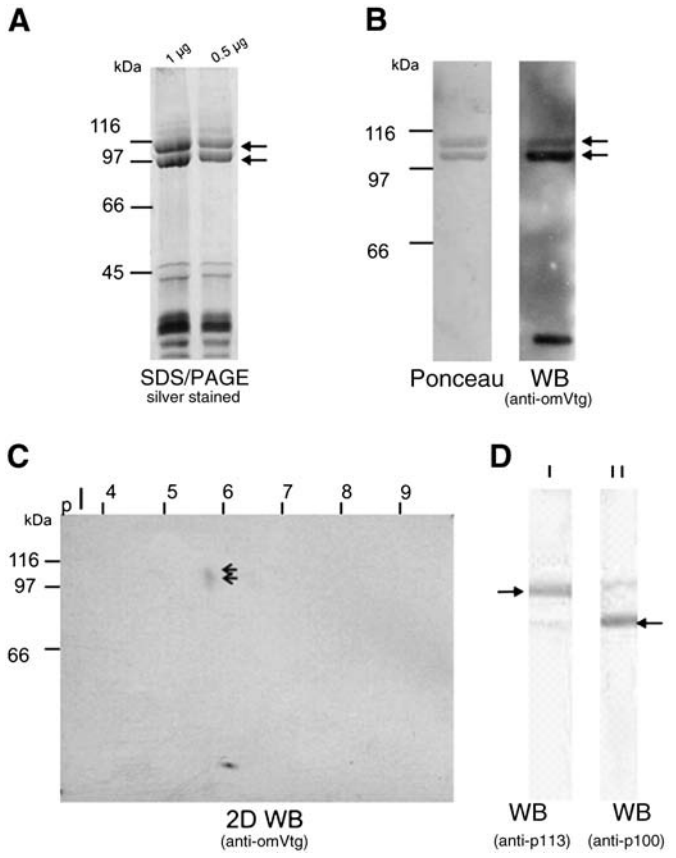

Fig. 1. Yolk proteins in *B. arenarum* oocytes and specific antibodies production. A. Oocytes extracts (1 and 0.5 µg of total protein) were analyzed on 8% gel under reducing and denaturing conditions and silver stained. B. and C. Western blot analysis of oocytes extracts using anti-Vtg antiserum from Mozambique tilapia (anti-omVtg, 1:2000). B. Oocyte extracts (1 µg) were loaded in an 8% SDS-PAGE and then transferred to a nitrocellulose membrane. Left blot represents the Ponceau staining of the membrane. C. Analysis of mature oocyte (1 µg total protein) by 2D gels. D. Specificity of anti-p113 and anti-p100 antiserums. Oocyte extracts (0.1 µg total protein) were analyzed in 10% SDS-PAGE and Western blots using anti-p113 (lane I) or anti-p100 (lane II) sera (1:1000 each). Black arrows show p113 and p100 and molecular mass markers (kDa) are displayed on the left.

2.3. Collagenase — dissociation of ovarian oocytes

Females were anesthetized and pieces of ovary were carefully dissected and incubated during 15 min in PBS buffer containing 4 mM EDTA, 25 mM sucrose and 1 mg/mL of collagenase.

2.4. Staging of B. arenarum oocytes

After collagenase treatment, oocytes, freed from follicular cells, were staged in accordance to Valdez Toledo and Pisanó (1980) as follows: stage I or previtellogenic *B. arenarum* oocytes (45–200 µm), stage II or primary vitellogenic (200–600 µm), stage III or late vitellogenic (600–1200 µm) and stage IV or full-grown (>1200 µm). The oocytes diameter was measured with a micrometer fitted into the eyepiece of a dissecting microscope. In some cases, ovarian oocytes were resuspended directly in Laemmli (1970) sample buffer prior to analysis by SDS-PAGE.

2.5. Protein analysis by 1D and 2D PAGE

Protein analysis by 1D SDS-PAGE was performed essentially according to the method of Laemmli (1970). Two dimension gel electrophoresis (2D PAGE) was performed on Protean IEF cell (Bio-Rad) using p*I* 3–10 strips (Amersham Biosciences) (first dimension). The strip was then rehydrated in buffer 8 M urea, 2% CHAPS and 50 mM dithiothreitol, and run in an 8% SDS-PAGE (second dimension). Gels were either stained with silver (Gradilone et al., 1998) or with Coomassie blue. Apparent molecular masses were estimated with molecular mass standards (Amersham Pharmacia Biotech, Piscataway,

Table 1
Analyses of *B. arenarum* yolk peptides by MS/MS spectrometry.

p133			
Peptide	Amino acid sequence	Homology	% of identity–**similarity**
1	TQVFAVSR	NH	
2	AEGiQEViR	B1	100–**100**
3	VQVDAVMAiR	A2 and B1	80–**90**
4	GYAAGASTDiFEFGVR	B1	44–**50**
p100			
Peptide	Sequence	Homology	% of identity–**similarity**
5	iQiSPYSQR	NH	
6	iiPiAiHYTR	NH	
7	SWiiQAiPVTR	NH	
8	VQETiMNVYMNR	NH	
9	GSQQAiQiAQDiiR	NH	
10	FiPGFSSSAQQiPVR	A2 and B1	86–**93**/66–**86**
11	iiYQMiHDGDiTNAEANR	B1	44–**61**

P113 and P100 were analyzed by MS/MS spectrometry. Table shows amino acid sequences of eleven abundant derivatized peptides from 1 to 11. The homology with *X. laevis* vitellogenin A2 (GenBank accession no. CAA68433) and B1 (GenBank accession no. NP_001094403) is presented. The percentages represent sequence identity (pain) and similarity (bold). Notice that peptide number 10 presented different percentages in the analysis with VtgA2 or Vtg B1. In De Novo sequencing i = I or L. NH indicates no homology.