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Dynamics of expression of two vitellogenin genes in the Chagas' disease vector *Triatoma infestans*: Analysis throughout pre-vitellogenesis and vitellogenesis

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ABSTRACT

The reproductive success of all oviparous species depends on vitellogenin (Vg) biosynthesis and its accumulation in the developing oocytes. The expression levels of two *Vg* genes (*Vg1* and *Vg2*) were analyzed by qPCR and western blot in fat body and ovaries of adult females, at different times after ecdysis (pre-vitellogenic phase) and after blood feeding of females (vitellogenic phase). *Vg* genes were also evaluated in fat bodies of adult males as well as in female fifth instar nymphs. No trace of *Vg* mRNA was detected in adult males or in nymphs. *Vg1* and *Vg2* were expressed in the fat bodies and ovaries of adult females. The *Vg* genes start to be expressed slightly in both tissues of adult females during pre-vitellogenesis. After blood feeding, *Vg1* and *Vg2* were up regulated and significant levels of *Vg* transcripts as well as protein expression were observed in fat bodies sampled throughout vitellogenesis. During this period however, the distribution patterns of *Vg1* and *Vg2* transcripts showed two peaks around early and advanced vitellogenesis (days 4 and 12 post-feeding, respectively). In the ovaries, levels of mRNAs increased from the day 10 post-blood feeding onwards. In addition, the immunofluorescence assays showed a strong signal for vitellin in the yolk bodies of terminal follicles of vitellogenic females. The involvement of fat body and ovary in the synthesis of Vg suggests different roles of Vgs in supporting the growth of oocytes.

1. Introduction

Chagas' disease, also known as American trypanosomiasis, is caused by the parasite *Trypanosoma cruzi*, which is transmitted to humans by vectors of the subfamily Triatominae (Hemiptera: Reduviidae). The disease and its vectors are extensively distributed from Southern United States of America to Southern Argentina and Chile (latitude 42°N to latitude 46°S). Currently, Chagas' disease is the fourth cause of economic losses through illness in Latin America, where about 8 million people are estimated to be infected with *T. cruzi* and more than 25 million people are at risk of contracting the infection (Rassi et al., 2010; World Health Organization, 2014).

All postembryonic stages of triatomine insects are hematophagous and at least one full blood meal is necessary for each molt. Therefore, these species may acquire the infection from their first blood meal (Lent and Wygodzinsky, 1979).

Triatoma infestans, one of the most important and widespread vectors of Chagas' disease in South America, has been the target of control programs as part of the Southern Cone Initiative (Moncayo and Silveira, 2009). However, the goals of current vector control policies are compromised by several factors including the abundance of other vector species and the extension of endemic areas, which hampers regularity in entomological surveillance (Tarleton et al., 2014). In addition, resistance to pyrethroid insecticides has been reported as a fact that renders difficult vector control strategies (Picollo et al., 2005; Lardeux et al., 2010; Gurevitz et al., 2012).

In insects, the vitellogenesis process is fundamental for egg development and therefore is a central event for their reproduction (Raikhel, 2005). During vitellogenesis, yolk protein

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precursors are produced in large amounts by extraovarian tissues, being vitellogenin (Vg) the main precursor protein of egg yolk. The fat body, a tissue hormonally regulated and functionally analogous to the vertebrate liver and adipose tissue, is the exclusive site of Vg synthesis in the majority of the insects, while in others, ovaries also synthesize Vgs (Melo et al., 2000; Giorgi et al., 2005; Bellés, 1998, 2005). The number of genes encoding insect Vgs varies from one to several in different species but in most insects, Vgs derive from a single *Vg* gene transcript of 6–7 kb (Tufail et al., 2014). Insect Vgs are large phospholipoglycoproteins of ~200 kDa with a remarkable degree of conservation in amino acid composition (Hughes, 2010). Vg synthesized in the fat body is secreted into the hemolymph and it is taken up by the developing oocytes by receptor-mediated endocytosis (Snigirevskaya and Raikhel, 2005; Tufail and Takeda, 2009). Following uptake by oocytes, Vgs are stored in specialized organelles or yolk bodies as vitellin (Vn). Vns provide raw materials for development of the embryos (Kunkel and Nordin, 1985). Although Vg is generally considered a female-specific protein, some male insects synthesize small amounts of Vg (Engelmann, 1979; Trenczek and Engels, 1986; Valle, 1993; Piulachs et al., 2003).

Hematophagous insects require a blood meal to activate numerous genes essential for digestion of the blood, synthesis of yolk protein precursors and ultimately, production of eggs (Raikhel, 2005). In the case of the vectors of Chagas' disease, the requirement of a blood meal is a key event that triggers the action of juvenile hormone for the initiation of vitellogenesis (Davey, 1997). Therefore, in these species, each gonotrophic cycle and egg development is coupled with the intake of blood (Friend et al., 1965; Stoka et al., 1987; Aguirre et al., 2008). However, autogeny, the capacity of an unfed adult female to develop eggs using blood ingested in the last nymphal stage, has been observed in some triatomines such as *Rhodnius prolixus* and *T. infestans* (Stoka et al., 1987; Noriega, 1992).

At present, great progress has been made in elucidating the nutritional control of vitellogenesis in anautogenous mosquitoes, particularly in *Aedes aegypti* (Attardo et al., 2005). On the contrary, in triatomines, the studies that focused on molecular mechanisms of blood meal regulation in the expression of essential genes for vitellogenesis are still scarce. Understanding the relationship between a blood requirement for the activation of *Vg* genes and egg development is of critical relevance in the biology of reproduction of triatomines. It is also an important way in the search of new tools for insect vector control. Recently, we have identified two Vg genes, *Vg1* and *Vg2*, in females of *T. infestans* (Blariza et al., 2014). The *Vg1* and *Vg2* mRNAs were found in fat bodies and ovaries of adult females. Expression levels of *Vg2* in the heads and thoracic muscles were low, while the *Vg1* transcript could not be detected in these two organs.

In order to better understand the molecular basis of blood regulation on vitellogenesis in Chagas' disease vectors, we have analyzed the expression of *Vg1* and *Vg2* genes at transcriptional and translational level in fat bodies and ovaries of anautogenous female adults of *T. infestans* at different times of pre-vitellogenic phase (unfed period post-ecdysis) as well as during the vitellogenic phase triggered after blood feeding. Additionally, transcription levels of Vgs were investigated in fat bodies of fifth-instar female nymphs and in adult males.

2. Materials and methods

2.1. Insects and tissue sampling

T. infestans was reared at 28 ± 1 °C at a relative humidity of 60–70% with a 6-h light/18-h dark cycle and fed once every two weeks on restrained chickens. Anautogenous females were obtained under our parameters of rearing. Fifth instar male and female nymphs were sexed by the differences described by Espinola (1966) and grouped separately. Seven days after ecdysis, the insects were fed and females were placed together with males in individual containers (each couple in one container). Mating was controlled by the deposited spermatophores.

Under our standardized laboratory rearing conditions, the period between eclosion to the adult stage and the first adult blood meal (pre-vitellogenic phase) is for *T. infestans* of approximately 7 days, the oviposition takes place between days 13–15 after blood feeding and is extended approximately until the days 22–23 post-blood meal (vitellogenic phase). The onset of the post-vitellogenic period was detected with the interruption of the oviposition. Afterwards, like in other triatomines, for a second batch of eggs a new intake of blood meal was needed (Stoka et al., 1987; Aguirre et al., 2011).

The expression analysis of two *Vg* genes previously identified, *Vg1* (GeneBank accession number KF915268) and *Vg2* (GeneBank accession number KF915267) (Blariza et al., 2014), was carried out in fat bodies of adult females at days 4 and 6 after ecdysis (unfed period) and at days 1–14, 17, 20, and 24 after blood feeding. Aditionally, ovaries from adult females during pre-vitellogenic (days 4 and 6) and vitellogenic (days 1–14 and 17) phases were also sampled. At the time of advanced vitellogenesis, fully developed oocytes free of outer sheaths were removed from terminal follicles before tissue processing. Studies were also performed in fat bodies sampled from fifth-instar females sampled from the day 5 to 15 after blood intake as well as from adult males throughout post-ecdysis (days 4 and 6) and post-feeding (days 1–14, 17, 20, and 24) periods.

For RNA extraction each sample was a pool of tissues from three adult specimens and five fifth instar nymphs. Fat bodies and ovaries were dissected under aseptic conditions and stored in liquid nitrogen until used for RNA extraction. For western blot assays, fat bodies and ovaries from five adult females were dissected under cold phosphate buffered saline (PBS, 6.6 mM Na_2HPO_4/KH_2PO_4, 150 mM NaCl, pH 7.4), pooled and homogenized as described elsewhere (Aguirre et al., 2008). Protein concentration was determined according to Bradford (1976), using bovine serum albumin (BSA) as standard.

2.2. Isolation of total RNA and cDNA synthesis

Total RNA was isolated from pools of insect tissues using MasterPure RNA Purification Kit (Epicentre, Madison, WI, USA) following the manufacturer's protocol and eluted in 33 μl of nuclease-free water. This kit includes a DNase treatment in order to eliminate potential genomic DNA contamination. The RNA concentration was determined by absorption at 260 nm.

First-strand cDNA synthesis was performed with 1 μl of Oligo-dT_{20} (50 μM) (Invitrogen, Carlsbad, CA, USA), 1 μg of total RNA, and 400 U of SuperScript III RT (reverse transcriptase, Invitrogen) in a 20 μl reaction volume incubated at 55 °C for 1 h.

2.3. Quantitative PCR (qPCR)

In order to determine the patterns of yolk protein gene expression, the transcript levels of *Vg1* and *Vg2* genes in different tissues, sexes, and development stages of *T. infestans* were measured by qPCR. Gene specific primers (Table 1) and Taqman probes were designed according to the corresponding cDNAs using the Primer Express program (Applied Biosystems, Foster City, CA, USA). Reverse transcription polymerase chain reaction (RT-PCR) analysis was used to verify that the PCR product, obtained with each specific primer pair, showed a single band of the expected size (71 bp). The PCR products corresponding to *Vg1*, *Vg2*, and β-*actin* were cloned into the pCR4-TOPO TA cloning vector (Invitrogen) and