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cDNA isolation and characterization of two vitellogenin genes in the Chagas' disease vector *Triatoma infestans* (Hemiptera, Reduviidae)

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ABSTRACT

Two vitellogenin genes (*Vg1* and *Vg2*) were identified in the Chagas' disease vector *Triatoma infestans*. The putative coding sequence corresponding to *Vg2* was found to be 5553 bp long, encoding 1851 amino acids in a single open reading frame. The comparative analysis of the deduced amino acid sequences from *Vg1* and *Vg2* cDNA fragments of *T. infestans* revealed 58.94% of identity with 76.43% of homology. The phylogenetic tree based on the complete Vg amino acid sequences of hemimetabolous insects unambiguously supported two clusters, one consisting of Vg sequences from dictyopteran and the other containing Vg sequences of hemipteran. The *Vg1* and *Vg2* mRNAs were detected in fat bodies and ovaries of adult females with the highest levels of both *Vg* transcripts in the first tissue. Quantitative PCR showed low expression of *Vg2* in head and muscle of adult females, while the *Vg1* transcript was not present in these organs. Neither *Vg1* nor *Vg2* was expressed in fifth instar nymph fat bodies or in adult male fat bodies, heads, and muscles.

1. Introduction

Chagas' disease (American trypanosomiasis) is the fourth cause of economic losses through illness in Latin America, with about 8 million persons infected and around 109 million living in endemic areas (Rassi et al., 2010). The disease is produced by infection with *Trypanosoma cruzi*, parasite transmitted by hematophagous insects of the subfamily Triatominae (Hemiptera, Reduviidae). The members of this subfamily are hemimetabolous insects with incomplete metamorphosis; the adults of both sexes and the five nymphal instars are bloodsuckers and at least one full blood meal is necessary for each molt. The subfamily Triatominae includes more than 140 species, most of which are actual or potential vectors of *T. cruzi*. Considering the different habitats described for the members of Triatominae, from exclusively sylvatic species to those well adapted to human dwellings, only a few, those with a high degree of adaptation to the domestic environment, have been recognized as effective vectors of trypanosomiasis in humans. Among them, *Triatoma infestans* is the most important and widespread vector of Chagas' disease in South America, where it has been the target of control programs as part of the Southern Cone Initiative. The interruption of transmission of this parasitic and infectious disease consists of vector control by insecticide treatment of infested dwellings. However, though Uruguay, Brazil, and Chile have been declared free of Chagas' disease transmission by *T. infestans* (Lorca et al., 2001; Silveira and Vinhaes, 1999; World Health Organization, 1994), high levels of *T. infestans* reinfestation after spraying were observed in Argentina, Bolivia, and Paraguay (Gürtler et al., 2007). Moreover, vector control failure caused by resistance to pyrethroid insecticides has been detected (Germano et al., 2010; Picollo et al., 2005; Santo Orihuela et al., 2008; Toloza et al., 2008; Vassena et al., 2000). Analyses of fundamental genes in these insect vectors as those related to their reproduction are of considerable importance and should be undertaken.

The majority of blood-feeding insects, including triatomines, ingest a large amount of blood in a single meal. The digested blood contains a large amount of amino acids and lipids that can be used to produce molecules such as vitellogenins (Vgs). Vg is the precursor protein of egg yolk vitellin (Vn) that provides energy reserves in oviparous vertebrates and invertebrates. In most insects, Vgs are synthesized exclusively in the fat body, while in others, the ovary is a complementary vitellogenic organ (Belles, 1998, 2005; Giorgi et al., 2005; Melo et al., 2000). In female insects, the main function of the fat body is to produce massive amounts of yolk protein precursors, which are secreted into the hemolymph and then sequestered by competent oocytes via receptor-mediated endocytosis (Snigirevskaya and Raikhel, 2005). During these processes, Vgs and Vns are modified by glycosylation, lipidation, phosphorylation and proteolytic cleavage (Dhadialla and Raikhel, 1990). Following uptake by oocytes, Vgs are stored in cytoplasmic granules as Vns providing raw materials for development of the embryos (Kunkel and

Abbreviations: bp, base pair(s); cDNA, DNA complementary to RNA; RNA, ribonucleic acid; mRNA, messenger RNA; DNase, deoxyribonuclease; dNTP, deoxyribonucleoside triphosphate; kb, kilobase(s) or 1000 bp; kDa, kilodalton(s); Oligo, oligodeoxyribonucleotide; *Vg*, vitellogenin gene; *Vgs*, vitellogenin genes; Vg, vitellogenin protein; Vgs, vitellogenin proteins; Vn, vitellin; Vns, vitellins.

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Nordin, 1985). Synthesis of Vg occurs in smaller amounts in males of some insect species (Engelmann, 1979; Piulachs et al., 2003; Trenczek and Engels, 1986; Valle, 1993).

Vg cDNAs have been isolated from several insect species (Tufail and Takeda, 2008). Insect Vgs are phospholipoglycoproteins encoded by mRNAs of 6–7 kb that are translated as primary products of ~200 kDa. These primary products have been characterized at a molecular level for Hemimetabola and Holometabola species (Sappington et al., 2002; Tufail et al., 2004). The amino acid sequence, structure, and composition of Vgs are sufficiently conserved between insects and other oviparous animals indicating an origin from a common ancestral protein (Sappington et al., 2002). The insect *Vgs* constitute a multigene family, with two or three members reported from a number of species (Hughes, 2010).

Here we report for the first time the nucleotide sequence and deduced amino acid sequence for a *Vg* in a triatomine species. We have identified two *Vg* genes (*Vg1* and *Vg2*) and have explored their expression patterns at the mRNA level in both sexes, different tissues, and two developmental stages.

2. Material and methods

2.1. Insects and tissue collection

T. infestans was reared at 28 ± 1 °C at a relative humidity of 60–70% with a 6-hour light/18-hour dark cycle and fed once every two weeks after molt on restrained chickens. For the experiments, fifth instar male and female nymphs were sexed by the differences described by Espinola (1966) and separated before feeding. Females and males were maintained segregated after emergence until they were able to have a blood meal (day 7 post-ecdysis). After feeding, females were placed together with males in individual containers (each couple in one container). Mated females were checked by observation of the spermatophore. For the *Vg* cDNA sequence identification, adult female fat bodies were extracted after 3–6 days of the blood feeding. Each sample was a pool of tissue from five adult female specimens. The expression analysis of the *Vg* genes was carried out using adult specimens and fifth instar nymphs after 4–5 days of feeding. Thoracic muscles, heads and fat bodies were collected from adult females and males. Additionally, ovaries from adult females and fat bodies from female nymphs of the fifth instar were also obtained. Each sample was a pool of tissue from three adult specimens and five fifth instar nymphs. The tissues were dissected under aseptic conditions and stored in liquid nitrogen until used for RNA extraction.

2.2. Isolation of total RNA and cDNA synthesis

Total RNA was extracted from pools of insect tissues using TRizol reagent according to the manufacturer's specifications (Invitrogen, Carlsbad, CA) and eluted in 20 μL of nuclease-free water. For quantitative PCR (qPCR) total RNA was isolated from pools of insect tissues using MasterPure RNA Purification Kit (Epicentre, Madison, WI) 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), 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. Amplification and sequencing of vitellogenin cDNA

Rapid amplification of 3′ cDNA end (3′-RACE) was performed using the GeneRacer commercial kit (Invitrogen). 1 μg of total RNA was reverse transcribed with GeneRacer Oligo-dT and Superscript III RT. A forward primer designed by Lee et al. (2000), based on the conserved amino acid sequence GL/ICG present near the C-terminus, and a GeneRacer 3′ primer (homologous to GeneRacer Oligo-dT Primer) (Table 1) were used to amplify the first-strand cDNA and to obtain the 3′ cDNA encoding for Vg. Only mRNA with a polyA tail was reverse transcribed and amplified using the polymerase chain reaction (PCR). After the PCR product was electrophoresed, a band corresponding to the expected size of approximately 500 bp was excised from the agarose gel and purified using QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). The PCR product was then sequenced in an ABI 3130XL automated DNA sequencer (Applied Biosystems, Foster City, CA). The comparative analysis of the cDNA fragment of *T. infestans* with cDNA sequences of the *Vg* gene of other insect species revealed that it was the 3′ end of this gene. Following initial identification of the *T. infestans Vg* gene, a conventional PCR was performed using a specific primer designed from the 5′ end of the 3′ cDNA partial sequence of *T. infestans Vg* gene and a degenerate or unspecific primer (Table 1) to amplify a longer fragment of *Vg* cDNA. To obtain sequences closest to

Table 1
Sequences of all PCR primers used in this study.

Name	Sequences	Function
PCR degenerate primers (for conserved cDNA isolation)		
PF1D	5′-GTNTTYGARCCNTAYYTNGARGGN-3′	Sense
RipF 2318	5′-CTTCAAMRNATGGCAGA-3′	Sense
C1	5′-CCBRTDGCCADDGGGAATCC-3′	Antisense
Unspecific primers (for RT-PCR)		
RhoF4766	5′-CATCAAGCCACAACTTTC-3′	Sense
RhoF4820	5′-GAAGGTGCTTCATATCAAAAGG-3′	Sense
FA5	5′-ACTCTCCATCGAATGTGCCGC-3′	Sense
FA12	5′-ACTTGGAACTGCAGGTAACTTGG-3′	Sense
RA1	5′-AGAGCACAGCAGTGCCTGTCCGGG-3′	Antisense
RA5	5′-AGACTGAATATGGTTATTCAGGCC-3′	Antisense
RA9	5′-CAACCGGATCTTCAGAAGTAGTTGG-3′	Antisense
RA12	5′-CCCAAGTTACCTGCAGTTCCA-3′	Antisense
Specific primers (for RT-PCR)		
PR1	5′-TCGGGATTCGCTCCTTTTCTTGT-3′	Antisense
PF2	5′-TGATGTAGAAGCCGGGCGTAACA-3′	Sense
PF3	5′-AAGTTTTAGATTTCTCTAAGGAATCAA-3′	Sense
PF2	5′-GTACAAATTTATGCCCTTCCCAACGG-3′	Sense
PR3	5′-CCGTTGGGAAGGGCATA-3′	Antisense
PF4	5′-AAGCCACAACTTTCGACAACAG-3′	Sense
PF5	5′-CCAGTAAATGTGGGTAAATGCTGG-3′	Sense
PR4	5′-GCATGCCAGCATTTACCCA-3′	Antisense
PF6iso	5′-CTGCCCGGGCTTTTACT-3′	Sense
PFA	5′-ATTTGTCGTTCAGATCAGCCCC-3′	Sense
PRA	5′-TAAACCAAGCGTTTGCCGGT-3′	Antisense
PFB3′	5′-TAAGCGAAGAAGAAGCTGGACG-3′	Sense
TIR7	5′-TATATCTGCCCATGAGATGGC-3′	Antisense
TIR9	5′-CCGTGAATGCAGTGAGAACGC-3′	Antisense
TIF8	5′-CAGAGTCTACCCCAGACTTGCC-3′	Sense
TIF9	5′-CAGAGAAACTGCACACAACCG-3′	Sense
Cloning		
M13F	5′-GTAAAACGACGGCCAG-3′	Sense
M13R	5′-CAGGAAACAGCTATGAC-3′	Antisense
RACE primers (for full cDNA isolation)		
I-6	5′-GGTATTTGCGG-3′ (Lee et al., 2000)	3′-RACE of Vg-1
PF5	5′-CCAGTAAATGTGGGTAAATGCTGG-3′	3′-RACE of Vg-2
Race5′	5′-TAGACTTAGAAATTAATACGACTCACTA TAGGCGCGCCACCG-3′	5′-RACE of Vg-2
GeneRacer3′	5′-GCTGTCAACGATACGCTACGTAACG-3′	3′-RACE of Vg-1
GeneRacer3′	5′-GCTGTCAACGATACGCTACGTAACG-3′	3′-RACE of Vg-2
TIR8	5′-CGTGATGGTTACTAGAGCAGGTCC-3′	5′-RACE of Vg-2
qPCR primers (for real time PCR)		
QAF5	5′-TATAATCAAGGATCCCTTAGAATTTGC-3′	*Vg-1, forward*
QAF2	5′-CGCATGAGAAGCTCTCTAACCA-3′	*Vg-2, forward*
QβactinaF	5′-CCCCTTTCAGTGAGGATCTTCA-3′	Internal control
QAR5	5′-TTGGCTGGTCCTTCACAAGTT-3′	*Vg-1, reverse*
QAR2	5′-ACGCGATGATTAGTGCATCCT-3′	*Vg-2, reverse*
QβactinaR	5′-CGCCATCCTTCGATTGGA-3′	Internal control