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Effects of chitin synthase double-stranded RNA on molting and oogenesis in the Chagas disease vector *Rhodnius prolixus*

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ABSTRACT

In this study, we provided the demonstration of the presence of a single *CHS* gene in the *Rhodnius prolixus* (a blood-sucking insect) genome that is expressed in adults (integument and ovary) and in the integument of nymphs during development. This *CHS* gene appears to be essential for epidermal integrity and egg formation in *R. prolixus*. Because injection of CHS dsRNA was effective in reducing *CHS* transcript levels, phenotypic alterations in the normal course of ecdysis occurred. In addition, two phenotypes with severe cuticle deformations were observed, which were associated with loss of mobility and lifetime. The CHS dsRNA treatment in adult females affected oogenesis, reducing the size of the ovary and presenting a greater number of atresic oocytes and a smaller number of chorionated oocytes compared with the control. The overall effect was reduced oviposition. The injection of CHS dsRNA modified the natural course of egg development, producing deformed eggs that were dark in color and unable to hatch, distinct from the viable eggs laid by control females. The ovaries, which were examined under fluorescence microscopy using a probe for chitin detection, showed a reduced deposition on pre-vitellogenic and vitellogenic oocytes compared with control. Taken together, these data suggest that the *CHS* gene is fundamentally important for ecdysis, oogenesis and egg hatching in *R. prolixus* and also demonstrated that the *CHS* gene is a good target for controlling Chagas disease vectors.

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1. Introduction

Rhodnius prolixus is one of the most important vectors of Chagas disease, a potentially life-threatening illness caused by the protozoan parasite *Trypanosoma cruzi*. Chagas disease is a serious public health problem primarily observed in Latin America. However, in the past decades, Chagas disease has been increasingly detected in the United States of America, Canada, many European countries and some Western Pacific countries (da Mota et al., 2012; Schmunis and Yadon, 2009). It has been estimated that 7–8 million individuals worldwide are infected (WHO, 2013). Because of the lack of a vaccine or an effective treatment for chronic Chagas disease, vector control is one of the primary methods for preventing this disease. In South and Central America, *R. prolixus* is the second most important vector of Chagas disease (da Mota et al., 2012; Schmunis and Yadon, 2009).

Chitin is a linear polymer of N-acetylglucosamine (GlcNAc), an essential element of the exoskeleton in insects. Chitin is also found in the tracheae, salivary glands (Kramer and Muthukrishnan, 2005) and peritrophic matrices (PM) of many insect species (Arakane et al., 2004; Hegedus et al., 2009; Ibrahim et al., 2000; Merzendorfer and Zimoch, 2003; Shao et al., 2001; Zhu et al., 2002; Zimoch and Merzendorfer, 2002). Recently, chitin has been described as a component in eggs of insects and is important for hatching in *Aedes aegypti*, *Tribolium castaneum* and *R. prolixus* (Arakane et al., 2008; Mansur et al., 2010; Moreira et al., 2007).

Chitin synthase (CHS) belongs to a large family of glycosyltransferase family 2 (GT2) enzymes. These membrane proteins are highly conserved in some chitin-synthesizing organisms (Coutinho et al., 2003; Saxena et al., 1995). The amino acid sequences of

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several insect CHSs contain three distinct domains: (1) an N-terminal domain containing transmembrane helices, which is less conserved; (2) a middle catalytic domain, which is highly conserved even among CHSs from different taxa comprises approximately 400 amino acids that contain three motifs, GEDRW, QRRRW and SWGTRE, that are regarded as CHS signature motifs; and (3) a multiple transmembrane segments located in the C-terminal domain (Kramer and Muthukrishnan, 2005; Merzendorfer and Zimoch, 2003).

Most insects possess two *CHS* genes. *CHS1* or *A* is involved in the synthesis of chitin in the cuticle and trachea and is expressed throughout the insect developmental cycle (Arakane et al., 2004, 2005; Tellam et al., 2000; Zhu et al., 2002). Alternative splicing of the *CHS1* gene has been described in some insects (Arakane et al., 2004, 2005; Ashfaq et al., 2007; Chen et al., 2007; Gagou et al., 2002; Hogenkamp et al., 2005; Qu and Yang, 2011; Wang et al., 2012; Zhang et al., 2010a). The second gene, *CHS2* or *B*, is responsible for the synthesis of chitin in the PM of the midgut and is expressed in the cells lining the guts of adults (Arakane et al., 2004, 2005; Ibrahim et al., 2000; Zimoch and Merzendorfer, 2002; Zimoch et al., 2005). No alternative splicing has been reported for *CHS2* in insects.

During its biological life cycle, *R. prolixus* experiences five nymphal instar before reaching adulthood. As a hematophagous insect, blood meals are essential in *R. prolixus* for ecdysis and egg production in adult females (Buxton, 1930). During ecdysis, chitin metabolism is increased to produce a new exoskeleton. The old exoskeleton is resorbed, and parts of the digested material will be recycled. The new cuticle is sclerotized, acquiring hardness and tan color characteristics (Kramer and Muthukrishnan, 2005). The molting cycle is achieved through chitin degradation enzymes, such as chitinases and β-N-acetylglucosaminidases (Fukamizo, 2000; Kramer et al., 1985; Kramer and Koga, 1986; Wilson and Cryan, 1997), and enzymes involved in chitin synthesis, such as CHS (EC: 2.4.1.16), which is responsible for the last step of chitin polymer formation (Glaser and Brown, 1957a, b; Kramer and Muthukrishnan, 2005; Merzendorfer and Zimoch, 2003).

The cuticle is a vital structure that protects insects from the environment. This protective covering prevents infection, mechanical injury and dehydration and facilitates locomotion, growth, respiration and communication (Kramer and Muthukrishnan, 2005). The cuticle consists of two principal covers: the outer layer, or epicuticle, consists of a lipoprotein layer followed by a polyphenol layer, which is overlaid with a wax coating, and the inner layer, or endocuticle, which consists of a protein matrix associated with chitin fibers (Hackman, 1953; Vavricka et al., 2011).

Oogenesis is a physiological process that occurs in three stages: pre-vitellogenesis, vitellogenesis and choriongenesis. Initally, the oocyte growth rate is slow during pre-vitellogenesis. In this stage, oocytes receive nutrients from nurse follicle cells directly through cytoplasmic cords (Büniing, 1994; Huebner, 1981; Huebner and Anderson, 1972). Later in oocyte development, there is a fast growth stage during vitellogenesis. In this stage, the oocyte accumulates large amounts of nutritive reserves that subsequently sustain embryo growth. During choriongenesis, the last stage of egg formation, the shell or chorion is formed, and this structure ensures the integrity of the eggs outside the mother's body, provides mechanical protection, prevents egg dehydration and facilitates fertilization and embryo respiration (Beament, 1946; Fakhouri et al., 2006; King and Aggarwal, 1965; Mazur et al., 1982; Telfer and Anderson, 1968; Waring and Mahowald, 1979).

Gene silencing using the RNAi technique is a powerful tool for investigating and developing an understanding of gene function in insects (Chen et al., 2010, 2008; Hannon, 2002). Gene silencing is achieved through the introduction of double-stranded RNA (dsRNA) into a wide variety of cells and organisms, resulting in the post-transcriptional depletion of endogenous mRNA or the inhibition of translation, a phenomenon known as RNA interference (Flores-Jasso et al., 2004; Sharp, 1999; Valdes et al., 2003).

Exoskeleton formation and egg production are indispensable elements for the evolutionary success of insect species; thus, the chitin molecule present in the cuticle and egg might represent an adequate target for vector control. Because chitin is absent in plants and vertebrates, the CHS genes have been suggested as specific target genes in biorational insect control studies (Arakane et al., 2005; Chen et al., 2007; Tian et al., 2009; Zhang et al., 2010b).

In this study, we provided the demonstration of the presence of a single *CHS* gene in the *R. prolixus* genome that is expressed in the integument and ovaries. The importance of *CHS* for cuticle integrity and egg formation in the blood-sucking insect *R. prolixus* was determined using RNAi. The injection of CHS dsRNA induced severe phenotypic alterations in the normal course of ecdysis, oogenesis and egg hatching, indicating that the *CHS* gene is a good target for controlling Chagas disease vectors.

2. Materials and methods

2.1. Animal maintenance and ethics statement

R. prolixus adults and nymphs were maintained at 28 °C and 80–90% relative humidity in the laboratory of Insect Biochemistry at the Institute of Medical Biochemistry at the Federal University of Rio de Janeiro, Brazil. The adult insects were fed rabbit blood at 3-week intervals; 1st, 2nd, 3rd and 4th instar nymphs were allowed to feed on rabbit at 4-week intervals; and 5th instar nymphs were fed rabbit blood at 5-week intervals. All animal care and experimental protocols were conducted according to the guidelines of the institutional care and use committee (Committee for Evaluation of Animal Use for Research from the Federal University of Rio de Janeiro, CAUAP-UFRJ, Brazil). The protocols were approved through CAUAP-UFRJ under registry #IBqM001.

2.2. R. prolixus genome analysis

R. prolixus CHS (*RpCHS*) was identified in the *R. prolixus* genome through a local tBlastn search (Altschul et al., 1997) using the *CHS1* sequence from *T. castaneum* (AY291475) as a query. This gene is located in the *R. prolixus* Contig 17687.30 whole genome shotgun sequence (GenBank ID: ACPB02029707). The open reading frame (ORF) were predicted by the ORF finder (http://www.ncbi.nlm.nih.gov/gorf/) and by Expasy translate (http://web.expasy.org/translate/) (Gasteiger et al., 2003) and were used as a strategy to design specific primers (Table 1) for the amplification of the *RpCHS* cDNAs through PCR.

2.3. R. prolixus ovary and integument CHS gene expression, sequence alignment and phylogenetic tree construction

To evaluate *CHS* expression in the ovaries, adult females were dissected at 48 h after the first blood meal, and the ovaries, free of tracheae and other tissues, were collected. The epidermal *CHS* expression was evaluated using integument from adult males (free of other tissues and the head). The tissues were homogenized in liquid nitrogen, and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. The RNA concentration was determined at 260 nm (Lee and Schmittgen, 2006) using a SmartSpect™ plus Spectrophotometer (Bio-Rad, Hercules, California, USA). RNA from the integument and the ovaries (1 μg each) was treated separately with 1 U of RNAse-free DNAse I (Fermentas, Burlington, Canada) for

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