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Citrus, a key insect eggshell protein

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ABSTRACT

Molecular aspects of chorion synthesis in insects have been studied deeply in species with meroistic ovaries. Information available in insects with panoistic ovaries is principally structural whereas molecular information in these species is scarce. This paper seeks to balance the above situation by describing a novel chorion gene, Citrus, from the cockroach *Blattella germanica*, a phylogenetically basal hemimetabolan insect with reproduction regulated by juvenile hormone and with panoistic ovaries. During previous work we discovered a series of novel genes which were specifically expressed during chorion formation in *B. germanica*. One of them, herein named Citrus, was peculiar due to its high copy number and its very transient expression. In the present paper we characterize Citrus in terms of structure and function. The most prominent structural feature is that the protein contains a motif which is repeated 33 times encompassing almost all the sequence. By using RNAi techniques we have demonstrated that Citrus is a key player in the building of the endochorion of *B. germanica* eggs.

1. Introduction

Chorion in insect eggs plays the essential function of protecting the embryo from external agents during development, while allowing gas exchange for respiration and the entry of sperm for fertilization (Woods et al., 2005; Zrubek and Woods, 2006). In insects, chorion synthesis is usually under the control of the 20-hydroxyecdysone synthesized by the follicular cells that surround the maturing oocyte (Belles et al., 1993). The follicular cells secrete the chorion components and are responsible for the final chorion structures which may be remarkably complex, as in Lepidoptera where chorion synthesis involves more than one hundred proteins (Margaritis, 1985a; Regier and Kafatos, 1985; Trougakos and Margaritis, 1998).

Insect chorion is structured into a number of layers which are conserved in their composition as well as in their disposition through diverse insect groups. In the cockroach *Blattella germanica* (Belles et al., 1993), as in the fly *Drosophila melanogaster* (Margaritis, 1985b), the vitelline membrane, the inner chorion layer, the endochorion and the exochorion are well defined layers. The vitelline membrane is the first layer secreted by the follicular cells. It is the most internal and interacts with the oocyte membrane. Covering the vitelline membrane there is the inner chorion layer, which is very faint but clearly distinguishable from the other chorion layers. The next to be deposited is the endochorion, which is the most complex and formed by an inner-endochorion, a columnar and an outer-endochorion layer. The last layer of the eggshell is the exochorion that gives the egg its final shape. The final step of choriogenesis is completed after oviposition, and consists in the hardening of the eggshell which occurs through a process of protein crosslinking.

Insect ovaries are classified into panoistic and meroistic types. In panoistic ovaries all oogonia are eventually transformed into oocytes; it is the most common type in phylogenetically basal insects, such as *B. germanica*. In meroistic ovaries, oogonia can derive into both oocytes and nurse cells; it is the most common in Paraneoptera and Endopterygota, and it has apparently evolved from an ancestral panoistic type (Büning, 1994). Although basic chorion information is available from a varied group of insects representing both ovary types (Beams and Kessel, 1969; Furneaux et al., 1969; Trougakos and Margaritis, 2002; Gaino et al., 2008), at the molecular level, these studies have greatly focused on Diptera and Lepidoptera, both having meroistic ovaries (Margaritis, 1985b; Spoerel et al., 1986; Cavaliere et al., 2008; Lecanidou and Papantonis, 2010).

In order to find regular patterns in the data available and to understand the molecular basis of the evolution of insect ovaries, molecular information on chorion synthesis in species with panoistic ovaries should be gathered. In this sense, and using a Suppression Substractive Hybridization (SSH) library approach (Irles et al., 2009a), we obtained a series of genes that are expressed

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differentially during the post-vitellogenic period of *B. germanica*, an hemimetabolan insect with panoistic ovaries, which encapsulates the eggs into an egg-case or ootheca, and whose reproduction is mainly regulated by juvenile hormone. From the SSH library, two genes were especially prominent, Brownie (Bg 30009) and Citrus (Bg30001). They were outstanding due to their remarkable abundance, their characteristic pattern of expression and because they had no homologues in insect gene databases. The function of Brownie has been recently described (Irles et al., 2009b) as encoding a protein that plays a key role in the formation of the sponge-like body, a complex structure of the eggshell that combines the aeropyle and the micropyle, and which is reminiscent of the *D. melanogaster* egg horns, that permit gas exchange between the egg and the exterior. The function of the other gene, Citrus, is reported in the present paper.

2. Material and methods

2.1. Animal sampling

Freshly emerged adult females of *B. germanica* were obtained from a colony reared in the dark at 29 ± 1 °C and 60–70% r.h. The length of the basal oocyte was used to stage the ovaries from 0- to 7-day-old, whereas the stages of choriogenesis (early chorion: EC, mid chorion: MC, and late chorion: LC) were determined according to the morphology of the anterior pole of the basal oocyte (Irles et al., 2009b). All dissections and tissue sampling were carried out on carbon dioxide-anaesthetized specimens.

2.2. Cloning of Citrus cDNA

A fragment of 2693 bp [accession number is FM253359.1] was previously obtained from a library of ESTs specific of post-vitellogenic ovaries (Irles et al., 2009b). To complete this sequence, 5′-rapid amplification of cDNA ends (RACE) was applied to RNA extracted from ovaries of 7-day-old females at the end of chorion formation, using FirstChoice® RLM-RACE (Ambion, Huntingdon, Cambridgeshire, UK) according to the manufacturer's instructions. The amplified fragment was analyzed by agarose gel electrophoresis, cloned into the pSTBlue-1 vector (Novagen, Madison, WI, USA) and sequenced. The sequences of all primers used are available on request.

2.3. RNA extraction and retrotranscription to cDNA

For Northern Blot analysis, total RNA was extracted from 7-day-old adult female ovaries at the end of chorion formation. For monitoring mRNA expression by qRT-PCR, total RNA was isolated from pools of four to six ovary pairs obtained in chosen ages and stages of the first gonadotrophic cycle. RNA extractions were performed using the Gen Elute Mammalian Total RNA kit (Sigma, Madrid, Spain). An amount of 400 ng from each RNA extraction was DNAse treated (Promega, Madison, WI, USA) and reverse transcribed with Superscript II reverse transcriptase (Invitrogen, Carlsbad CA, USA) and random hexamers (Promega). RNA quantity and quality was estimated by spectrophotometric absorption at 260 nm in a Nanodrop Spectrophotometer ND-1000® (NanoDrop Technologies, Wilmington, DE, USA).

2.4. Northern blot analysis

Total RNA (10 μg) was subjected to electrophoresis in 1% agarose gels containing formaldehyde, following the methodology previously described (Irles et al., 2009b). As a probe, a Citrus fragment of 213 bp was amplified by PCR and labelled with fluorescein using the Gene Images Random Prime-Labelling Module (GE Healthcare, Madrid, Spain).

2.5. Expression studies

Quantitative RT-PCR (qRT-PCR) was used to study Citrus expression during the first gonadotrophic cycle and to assess the effect of RNAi over mRNA levels. PCR primers used in qRT-PCR expression studies were designed using the Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA). The BgActin-5c gene of *B. germanica* (accession number is AJ862721) was used as a reference. qRT-PCR reactions were performed and analyzed as previously reported (Irles et al., 2009a). Statistical analysis of gene expression values was carried out using the REST 2008 program (Relative Expression Software Tool V 2.0.7; Corbett Research) (Pfaffl et al., 2002). This program makes no assumptions about the distributions, evaluating the significance of the derived results by Pair-Wise Fixed Reallocation Randomization Test tool in REST (Pfaffl et al., 2002).

2.6. RNAi experiments

A dsRNA encompassing a 213 bp region starting at nucleotide 1650 of Citrus sequence (Fig. S1) labelled as dsCitrus-1 was prepared, then amplified by PCR and cloned into pSTBlue-1 vector. A non-coding sequence from the pSTBlue-1 vector (dsMock) was used as control dsRNA. The dsRNAs were prepared as previously described (Ciudad et al., 2006). Five-day-old adult females were injected into the abdomen with 1 μg of dsCitrus-1 in a volume of 1 μl. Control specimens were injected with the same volume and dose of dsMock. An alternative dsRNA (dsCitrus-2), encompassing a 309 bp region starting at nucleotide 211 of Citrus sequence (Fig. S1), was prepared following the same methodology, and used to asses the specificity of the effects obtained with dsCitrus-1.

2.7. Scanning electron microscopy (SEM)

Selected eggs from dsCitrus-1- and dsMock-treated females were fixed in 2.5% glutaraldehyde in cacodylate buffer 0.2 M for at least 2 h. After rinsing twice with the same buffer, the samples were then treated with 1% osmium tetroxide (Ted Pella, Inc, Redding, USA) at 4 °C for 1 h. The tissues were dehydrated with increasing concentrations of alcohol at 15 min intervals. Finally, the samples were subjected to critical-point drying in order to complete the dehydration process. The samples were attached to stubs with double-stick tape and coated with gold-palladium in a sputter-coating apparatus and observed with a Hitachi S-3500N scanning electron microscope at 5 kV (Hitachi High-Technologies Corporation, Tokyo, Japan). After fixation, oocytes were gently ripped with a microforceps in order to expose the layers.

2.8. Transmission electron microscopy (TEM)

Ovarian follicles at the end of choriogenetic period, from both dsCitrus-1 and dsMock-treated females, were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 100 mM phosphate buffer (PB, pH 7.4) for 2 h and rinsed 4 times with 100 mM PB. Samples were then postfixed in 1% osmium tetroxide (TAAB, Berks, UK) containing 0.8% of potassium hexacyanoferrate (III) (Sigma) for 2 h and washed with 100 mM PB. All these steps were made at 4 °C. Samples were dehydrated through a graded acetone series, infiltrated in Epon resin and polymerized for 48 h at 60 °C. Ultrathin sections were mounted in copper grids (200 mesh), contrasted with uranyl acetate and lead citrate solutions, and observed in a transmission electron microscope Jeol JEM-1400 (Jeol LTD, Tokyo,