Insect Biochemistry and Molecular Biology 42 (2012) 491–498

Contents lists available at SciVerse ScienceDirect

Insect Biochemistry and Molecular Biology

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

FoxO inhibits juvenile hormone biosynthesis and vitellogenin production in the German cockroach

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ARTICLE INFO

Article history:
Received 10 January 2012
Received in revised form
21 March 2012
Accepted 26 March 2012

Keywords:
FoxO
Insulin
Juvenile hormone
Vitellogenin
Blattella germanica
Nutritional signalling

ABSTRACT

The transcription factor Forkhead-box O (FoxO) is the main transcriptional effector of the Insulin Receptor/Phosphatidylinositol 3-kinase (InR/PI3K) pathway. In a situation of nutrient restriction, the pathway is inactive and FoxO translocates to the nucleus to exert its transcriptional action. In starved females of the cockroach *Blattella germanica*, the reproductive processes, and in particular the synthesis of juvenile hormone in the corpora allata and that of vitellogenin in the fat body, are arrested. In the present report we examine the possible role of FoxO in the transduction of the nutritional signals to these reproductive events. We first cloned FoxO cDNA from *B. germanica* (BgFoxO), and showed that its expression is not nutritionally regulated. BgFoxO knockdown using systemic RNAi *in vivo* in starved females elicited an increase of juvenile hormone biosynthesis, although without modifying mRNA levels of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase-1, HMG-CoA synthase-2, HMG-CoA reductase or methyl farnesoate epoxidase (CYP15A1) in corpora allata. In addition, BgFoxO RNAi treatment produced a remarkable increase of vitellogenin mRNA levels in fat body and of vitellogenin protein in the haemolymph. Our results indicate that BgFoxO plays an inhibitory role on juvenile hormone biosynthesis and vitellogenin production in a situation of nutrient shortage.

1. Introduction

Insect vitellogenesis involves coordinated activities between corpora allata (CA), fat body and ovaries. In many insect species, juvenile hormone (JH) synthesized and secreted by the CA activates vitellogenin (Vg) production in the fat body and its incorporation into the maturing oocytes (Belles, 2005). The regulation of JH synthesis and vitellogenin production includes different factors as neuropeptides, biogenic amines and nutritional signals, among others (Rauschenbach et al., 2004; Belles, 2005; Maestro et al., 2009). In the adult females of the cockroach *Blattella germanica*, JH biosynthesis shows a cyclic pattern, with a steady increase after the adult moult and a sudden decrease at oviposition time, remaining at low levels during the period of ootheca transport, and initiating a new gonadotrophic cycle after the eclosion of the nymphs from the ootheca (Maestro et al., 1994).

As a typical anautogenous species, the ingestion of a meal is a necessary requirement for initiating vitellogenesis in *B. germanica*. Thus, *B. germanica* starved females produce very low levels of JH and the Vg production is extremely reduced, whereas re-feeding triggers the vitellogenic processes (Osorio et al., 1997; Maestro et al., 2009). One of the signalling pathways involved in transducing the nutritional signals is the “target of rapamycine” (TOR) pathway. Knock-down of *TOR* expression produces a severe inhibition of JH production in adult females of *B. germanica* (Maestro et al., 2009). In addition, these females show a reduction of Vg mRNA in the fat body, not only derived from the reduced JH levels, but also due to a lack of a positive effect of TOR on *Vg* transcription (Maestro et al., 2009).

Besides TOR, other mechanisms have been related to nutritional signalling, particularly the Insulin Receptor/Phosphatidylinositol 3-kinase (InR/PI3K) pathway. Insect insulin-related peptides (IRP), analogously to vertebrate insulin, are secreted in response to different factors, especially high nutritional levels (Masumura et al., 2000; Ikeya et al., 2002; Geminard et al., 2009). Insulin receptor-mediated activation of PI3K increases the production of phosphatidylinositol trisphosphate (PIP_3) that, acting as a second messenger, recruits Akt to the plasma membrane. Once in the plasma membrane, Akt is phosphorylated and, in turn, it phosphorylates a number of downstream targets that ultimately carry out pathway effects (Puig et al., 2003; Baker and Thummel, 2007). One of the proteins phosphorylated by Akt is the transcription factor Forkhead-box, class O (FoxO), which has been revealed as the

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doi:10.1016/j.ibmb.2012.03.006

most relevant agent for the transcriptional activities of the InR/PI3K pathway (Murphy et al., 2003; Gershman et al., 2007).

FoxO proteins are a subgroup of the Forkhead-box family of transcription factors. This family comprises a large and diverse group of proteins characterized by a conserved DNA-binding domain (the Forkhead-box or Fox), classified from FoxA to FoxS on the basis of sequence similarity. Members of the class "O" share the characteristic of being regulated by the InR/PI3K signalling pathway by Akt phosphorylation at three conserved residues (Barthel et al., 2005; Greer and Brunet, 2005). This phosphorylation leads to the export of FoxO protein from the nucleus to the cytoplasm (Greer and Brunet, 2005; Baker and Thummel, 2007). Feeding condition, thus, maintains FoxO inactive in the cytoplasm, whereas starvation promotes its transcriptional activities upon nutritionally regulated genes in the nucleus.

In *B. germanica*, the mRNA levels of some genes related to reproductive processes are modified in starvation. mRNA levels of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase-1 and -2 and HMG-CoA reductase, enzymes of the mevalonate pathway but also involved in JH synthesis in the CA, are inhibited during starvation (Maestro et al., 2009). In addition, *Vg* expression in fat body is also reduced in starved females (Maestro et al., 2009). In this study, we analyzed the function of FoxO in *B. germanica* adult females, and in particular its relationship with the processes related to reproduction.

2. Materials and methods

2.1. Insects

Specimens of *B. germanica* were obtained from a colony reared with dog chow and water, in the dark at 30 ± 1 °C and 60–70% relative humidity. For the study of gene expression levels during the first gonadotrophic cycle, virgin females were used. For the RNAi experiments, we induced a second gonadotrophic cycle by removing the ootheca at the twelfth day of its transport period. For the starvation experiments, animals received only water after the imaginal moult or after the induction of the second gonadotrophic cycle. Dissections of CA and abdominal fat body were carried out on carbon dioxide-anesthetized specimens.

2.2. Cloning of BgFoxO cDNA

Degenerated primers based on conserved regions of insect and vertebrate FoxO sequences were used to obtain a *B. germanica* homologue cDNA fragment by RT-PCR. The first PCR amplification was carried out using cDNA generated by reverse transcription from RNA of UM-BGE-1 cells (derived from early embryos of *B. germanica*) as a template. Primers can be found as Supplementary data, Table 1. We amplified a 101 bp fragment, which was subcloned into the pSTBlue-1 vector (Novagen; 70596) and sequenced. This was followed by 3′-RACE and several 5′-RACEs (5′- and 3′-RACE System Version 2.0; Invitrogen) using different specific primers to complete the sequence. We used again cDNA from UM-BGE-1 cells as a template. PCR products were subcloned into the pSTBlue-1 vector and sequenced in both directions.

2.3. Phylogenetic analyses

We used the sequences from the following insects: *Drosophila melanogaster* (GenBank™ Accession Number: NP_996204), *Aedes aegypti* (ABK76646), *Anopheles gambiae* (CAD27476), *Nasonia vitripennis* (XP_001607658), *Apis mellifera* (XP_001122804), *Tribolium castaneum* (XP_975200), *Pediculus humanus* (XP_002425837) and *Acyrthosiphon pisum* (XP_001944722); the Nematoda: *Caenorhabditis elegans* (AAC47803); the Echinodermata: *Strongylocentrotus purpuratus* (ABB89484); and the Chordata: *Ciona intestinalis* (NP_001071717), *Gallus gallus* FoxO 3 (XP_001234496), *Xenopus laevis* FoxO 3 (AAI70411), *Homo sapiens* FoxO 1 (AAH70065), *H. sapiens* FoxO 3 (NP_001446) and *H. sapiens* FoxO 4 (NP_005929). The tree was rooted in the divergence between protostomes and deuterostomes. Protein sequences were aligned using ClustalX (Thompson et al., 1997). Poorly aligned positions and divergent regions were eliminated using Gblocks 0.91b (Castresana, 2000). The obtained alignment was analyzed with the PHYML 3.0 program (Guindon and Gascuel, 2003), based on the maximum-likelihood principle. Four substitution rate categories optimizing the gamma shape parameter were used. The data sets were bootstrapped for 100 replicates.

2.4. RNA extraction, cDNA synthesis and real-time PCR analyses

The CA and fat body expression levels of the different studied genes were analyzed using real-time PCR. cDNA was synthesized from total RNA as previously described (Maestro and Belles, 2006). An amount of 0.5 μg of total RNA was used in the case of fat bodies, whereas in the case of CA, the whole RNA from one pair of glands was used. The absence of genomic contamination was checked using a control without reverse transcription.

cDNA levels were quantified by using iQ SYBR Green supermix (Bio-Rad) in an iQ cycler and iQ single colour detection system (Bio-Rad) as previously described (Maestro et al., 2010). Primer sequences to amplify BgFoxO, HMG-CoA synthase-1 and -2, HMG-CoA reductase, methyl farnesoate epoxidase (CYP15A1), Vg and Actin 5C (used as a reference), are reported as Supplementary data, Table 1. Total reaction volume was 20 μl. All reactions were run in duplicate or triplicate. The program used to amplify the reaction was as follows: (i) 95 °C for 3 min; (ii) 95 °C for 10 s; (iii) 60 °C for 1 min; and (iv) repeat to step ii for 50 cycles. Real-time data were collected by iQ5 optical system software v. 2.0 (Bio-Rad).

2.5. RNA Interference

Systemic RNAi *in vivo* in females of *B. germanica* was performed as previously described (Maestro et al., 2009). Two different fragments, a 570-bp dsRNA fragment (dsFoxO) encompassing most of the Forkhead-box DNA binding domain and the 3′-contiguous sequence, and a 298-bp fragment (dsFoxO II) encompassing the protein C-terminus and part of the 3′ non-coding region, spanning positions 868 to 1437 and 1918 to 2215, respectively, of the BgFoxO cDNA, were used to generate two different dsRNA (Fig. 1B). As a control, a heterologous 307-bp fragment from the polyhedrin of *Autographa californica* nucleopolyhedrovirus (dsMock) was used (Maestro et al., 2011). To be confident that the treatment produced a sufficient BgFoxO reduction, we performed long time treatments. They consist in the injection of 2 μg of the dsRNA fragment into the abdomen of females in the first day of ootheca transport. Twelve days later, we removed the ootheca, triggering the beginning of a second gonadotrophic cycle in all respects similar to the first one. Dissections were carried out 5 days later. In addition, using this experimental model we avoid the possible developmental phenotypes caused by RNAi treatment at the nymphal stages and only concentrate in the effects of BgFoxO RNAi treatment in the adult. BgFoxO, HMG-CoA synthase-1 and -2, HMG-CoA reductase, CYP15A1, Vg, and Actin 5C (used as a reference) mRNA levels were determined by RT-qPCR.