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A polydnavirus-encoded ANK protein has a negative impact on steroidogenesis and development

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ABSTRACT

Polydnaviruses (PDV) are viral symbionts associated with ichneumonid and braconid wasps parasitizing moth larvae, which are able to disrupt the host immune response and development, as well as a number of other physiological pathways. The immunosuppressive role of PDV has been more intensely investigated, while very little is known about the PDV-encoded factors disrupting host development. Here we address this research issue by further expanding the functional analysis of *ankyrin* genes encoded by the bracovirus associated with *Toxoneuron nigriceps* (Hymenoptera, Braconidae). In a previous study, using *Drosophila melanogaster* as experimental model system, we demonstrated the negative impact of *TnBVank1* impairing the ecdysone biosynthesis by altering endocytic traffic in prothoracic gland cells. With a similar approach here we demonstrate that another member of the viral *ank* gene family, *TnBVank3*, does also contribute to the disruption of ecdysone biosynthesis, but with a completely different mechanism. We show that its expression in *Drosophila* prothoracic gland (PG) blocks the larval-pupal transition by impairing the expression of steroidogenic genes. Furthermore, we found that *TnBVank3* affects the expression of genes involved in the insulin/TOR signaling and the constitutive activation of the insulin pathway in the PG rescues the pupariation impairment. Collectively, our data demonstrate that *Tn*BVANK3 acts as a virulence factor by exerting a synergistic and non-overlapping function with *Tn*BVANK1 to disrupt the ecdysone biosynthesis.

1. Introduction

Parasitic wasps develop on a wealth of insect species, on which they induce a number of physiological and developmental alterations, which are essential to create a suitable environment for the development of their progeny (Pennacchio and Strand, 2006). These changes are currently denoted as host regulation, which is a complex process mediated by a network of molecular interactions, triggered and controlled by factors produced and released into the host by the ovipositing females (i.e. venom, microbial symbionts, ovarian secretions) and/or by the embryo (i.e. teratocytes, cells deriving from the dissociation of the embryonic membrane) or larvae (Pennacchio and Strand, 2006). Among microbial symbionts, polydnaviruses (PDVs) are potent immunosuppressive agents associated with ichneumonid and braconoid wasps parasitizing larval stages of moth larvae, and able to induce a number of pathological alterations in the host (Pennacchio and Strand, 2006; Strand and Burke, 2015). PDVs are integrated as proviruses in the wasp genome and replicate only in the epithelial cells of the ovarian calyx to produce free virions that are injected into the host at the oviposition. During this process they infect and express virulence factors in several host tissues, without undergoing replication (Herniou et al., 2013; Strand and Burke, 2015). The segmented genome of PDVs consists of multiple circles of DNA of different size, characterized by large non-coding segments and by genes showing a eukaryotic structure, often organized in gene families (Herniou et al., 2013; Strand and Burke, 2015). One of the most widespread gene family encodes ankyrin motif proteins (ANK), which are virtually expressed in all host tissues and found associated with a number of different pathological symptoms, ranging from immune to developmental alterations (Falabella et al., 2007; Strand and Burke, 2013). The viral ANK proteins have

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sequence similarity with members of IκB protein family, which control the NF-κB signaling in insects and vertebrate innate immunity (Silverman and Maniatis, 2001). Due to the lack of the regulatory sequences needed for their signal-induced and basal degradation, these ANK proteins appear to irreversibly bind to host NF-κB factors and block their transcriptional activity. Therefore, a function as suppressors of the host immune system has been proposed and demonstrated for some members of PDV *ank* genes (Thoetkiattikul et al., 2005; Falabella et al., 2007; Bitra et al., 2012). In contrast, we know comparatively much less on the role of *ank* genes, and more in general of PDV-encoded factors, in the induction of host developmental alterations.

The host-parasitoid association *Heliothis virescens-Toxoneuron nigriceps* (Lepidoptera, Noctuidae - Hymenoptera, Braconidae) provides a valuable experimental model system to study the molecular bases of developmental arrest of mature larvae, which is due to a combined action of PDV and teratocytes, disrupting the biosynthetic activity of prothoracic glands (Pennacchio et al., 1997, 1998) and the ecdysteroid metabolism (Pennacchio et al., 1994a) respectively. Since the ecdysone biosynthesis is well conserved in insects (Niwa and Niwa, 2014), to identify whether *Tn*BV genes can disrupt this biosynthetic pathway, we took advantage of the *Drosophila melanogaster* model system that allow to design experiments that are not doable in *Heliothis*. The powerful molecular genetics techniques that can be applied in *Drosophila* (del Valle Rodriguez et al., 2011) allow the study of the effect that the expression of virulence genes has on specific tissues during development. Indeed, this model system has been even employed for studying human viral pathogens (Hughes et al., 2012).

Using this approach, in our previous work, we have gained insights on the role of a member of the viral *ank* gene family of *Tn*BV, *TnBVank1* (Duchi et al., 2010; Valzania et al., 2014). We found that it functions as a virulence gene disrupting ecdysteroidogenesis in prothoracic gland by interfering with the endocytic trafficking of steroidogenic cells (Valzania et al., 2014). *Tn*BV genome carries two other members of the *ank* gene family (Falabella et al., 2007). In the present study we analyzed the effect of the expression of the *TnBVank3* in *Drosophila* steroidogenic cells. We found that also this gene contributes to the disruption of ecdysone biosynthesis by altering the expression of steroidogenic genes.

2. Materials and methods

2.1. Fly strains

Fly stocks were raised on standard cornmeal/yeast/agar medium at 18 °C. *y* w^{67c23} was used as the wild type stock in this study. We used the following Bloomington stocks: #5138 (y^1 w^*; *P[tubP-Gal4]LL7/TM3,* Sb^1 Ser^1); #7019 (w^*; *P[*w^{+mC}*=tubP-Gal80*^{ts}*]20; TM2/TM6B,* Tb^1); #8263 y^1 w^{1118}*; P[UAS-InR.A1325D]2. phm-Gal4* (Ono et al., 2006) was a gift from C. Mirth (*phm-Gal4,UAS-mCD8:GFP/TM6B*).

2.2. Crosses

For the *tub-Gal80*^{ts}; *phm-Gal4* experiments, *tub-Gal80*^{ts}*; phm-Gal4/TM6B* females were crossed at 21 °C to *UAS-TnBVank3* males, or to *y* w^{67c23} males as control. Larvae were raised at 21 °C and transferred at 29 °C at specific time points after egg laying (AEL). For the *UAS-InR*^{CA} expression, *tub-Gal80*^{ts}*; phm-Gal4/TM6B* females were crossed at 21 °C to *UAS-InR*^{CA}*;UAS-TnBVank3* males, and to *UAS-InR*^{CA} males as control. Larvae were raised at 21 °C and transferred at 29 °C after 3 days AEL.

2.3. Generation of TnBVank3-HA-Myc transgenic line

A construct containing the epitope tags hemagglutinin (*HA*) and *Myc* at the 3' end of *TnBVank3* gene was produced (Biomatik) and cloned into the pUAST-attb vector (Bischof et al., 2007). The transgenic *Drosophila* line carrying the *UAS-TnBVank3-HA-Myc* chimeric gene was obtained by phiC31 integrase-mediated insertion into the attP2 landing-site locus on the third chromosome by BestGene Inc (USA).

2.4. Immunofluorescence microscopy

Immunostaining on ring glands was performed as described previously (Valzania et al., 2016). The *Tn*BVANK3-HA-Myc protein was detected using a polyclonal rabbit anti-HA 1:50 (Santa Cruz Biotechnology, USA) and anti-rabbit Cy3-conjugated 1:2000 (Invitrogen, USA). The glands were mounted in Fluoromount G (Electron Microscopy Sciences, USA) and analyzed with TCS SL Leica confocal system. Images were processed using Adobe Photoshop CS6.

2.5. Protein extracts and western blot analysis

UAS-TnBVank3-HA-Myc or control *y* w^{67c23} males were crossed at 25 °C to *tub-Gal4/TM3*. Third instar larvae were collected and the total protein extraction and blot analysis were performed as already described (Romani et al., 2016). The *Tn*BVANK3-HA-Myc protein was detected using a monoclonal mouse anti-HA 1:100 (Santa Cruz Biotechnology, USA) and ECL Plex anti-mouse Cy3 1:2500 (GE Healthcare, USA).

2.6. 20-E rescue experiments

Two groups of ten *tub-Gal80*^{ts}; *phm-Gal4/UAS-TnBVank3* larvae, initially raised at 21 °C for 3 days AEL and then transferred at 29 °C for other 3 days were collected and placed in new tubes with yeast paste supplemented with 20-hydroxyecdysone (Sigma) 1 mg/ml and kept at 29 °C. As a control the same experiments were carried out on larvae of the same genotype fed with yeast paste containing an equal amount of ethanol.

2.7. Quantitative real-time PCR (qRT-PCR)

For *E74A*, *E75A* and steroidogenic gene expression experiments, total RNA was isolated from 3 independent biological samples of 5 larvae or prepupae. Total RNA was isolated using TRIzol reagent (Thermo Scientific), and DNA was removed by RNase-Free DNase Set (Ambion). qRT-PCR was performed on an ABI PRISM 7900 Real-Time PCR system (Applied Biosystems) by means of the Power SYBR-Green RNA-to-Ct-1-Step Kit (Applied Biosystems).

For the expression analysis of insulin and TOR pathway components, 15 brain-ring gland complexes (BRGCs) were dissected, in PBS buffer, from four independent biological samples. Total RNA was isolated using TRIzol reagent (Thermo Scientific), and contaminant DNA was removed by RNase-Free DNase Set (Ambion). cDNA synthesis was carried out with dT-primed M-MLV Reverse Transcriptase (LifeTechnologies). Quantitative PCR was carried out with FastStart SYBR Green Master Mix (Roche) on a QuantStudio 6 real-time thermal cycler.

The qRT-PCR primers used are listed in Table S1 in the supplementary material. For all of the genes examined, the reactions were conducted in technical triplicates. All transcript expression values were normalized to *Rpl23* gene.

2.8. Prothoracic gland size measurements

For measurements of the PG area, confocal images of PGs taken at 40× magnification were quantified with Photoshop CS6.

2.9. Statistical analysis

GraphPad Prism software was used for statistical analysis. Statistical significance was determined on the basis of unpaired *t*-test performed on the means and p values were calculated (* = $p < 0.05$;