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Knockdown of a Mosquito Odorant-binding Protein Involved in the Sensitive Detection of Oviposition Attractants

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Abstract Odorant-binding proteins (OBPs) were discovered almost three decades ago, but there is still considerable debate regarding their role(s) in insect olfaction, particularly due to our inability to knockdown OBPs and demonstrate their direct phenotypic effects. By using RNA interference (RNAi), we reduced transcription of a major OBP gene, CquiOBP1, in the antennae of the Southern house mosquito, Culex quinquefasciatus. Previously, we had demonstrated that the mosquito oviposition pheromone (MOP) binds to CquiOBP1, which is expressed in MOP-sensitive sensilla. Antennae of RNAi-treated mosquitoes showed significantly lower electrophysiological responses to known mosquito oviposition attractants than the antennae of water-injected, control mosquitoes. While electroantennogram (EAG) responses to MOP, skatole, and indole were reduced in the knockdowns, there was no significant difference in the EAG responses from RNAi-treated and water-injected mosquito antennae to nonanal at all doses tested. These data suggest that CquiOBP1 is involved in the reception of some oviposition attractants, and that high levels of OBPs expression are essential for the sensitivity of the insect’s olfactory system.

Keywords RNA interference · Culex quinquefasciatus antennae · CquiOBP1 · EAG · Oviposition attractants · MOP · Skatole · Indole

Introduction

Odorant binding proteins (OBPs) were identified almost three decades ago (Vogt and Riddiford 1981), but their roles in insect olfaction are still a matter of considerable debate. That OBPs are involved in odorant reception was disputed after odorant receptors (ORs) were demonstrated to respond to semiochemicals when expressed in heterologous systems. These expression systems, however, have limitations in addressing the role(s) of OBPs in olfaction. The heterologous expression system that uses Drosophila empty neurons (Dobritsa et al. 2003) includes surrogate OBPs, i.e., OBPs expressed in the ab3 sensilla, whereas in non-insect cell systems1 (Forstner et al. 2009) odorants are solubilized with organic solvent or with the addition of recombinant OBPs. Thus, ultimately the role(s) of OBPs in insect olfaction must be addressed by examining insects with reduced levels (knockdowns) or devoid of a test OBP (knockouts). In Drosophila, analysis of a mutant defective for expression of an OBP revealed that DmelOBP76a (aka LUSH) is required for the activation of pheromone sensitive neurons by (E)-11-vaccenyl acetate and associated behavior (Xu et al. 2005), but other insect species are not amenable to this type of genetic manipulation. Previously, we employed the empty neuron system of Drosophila to express the pheromone receptor from the silkworm moth, Bombyx mori, BmorOR1 alone or co-expressed with a pheromone-binding protein, BmorPBP1 (Syed et al. 2006). Despite the low levels of BmorPBP1 expression in this heterologous system, we demonstrated clearly that PBPs enhance the sensitivity of the insect olfactory system (Syed...

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1 We apologize for not being able to cite all the relevant literature due to reference limitations of a rapid communication.
of reception of oviposition attractants. Mosquitoes injected (RNAi) experiments to examine its function in the study, we used CquiOBP1 as a target in RNA interference sensitive to this pheromone (Leal et al. 2008). In the present dependent manner and to be expressed in antennal sensilla pheromone (MOP) (Laurence and Pickett 1982) nap H CquiOBP1 was shown to bind a mosquito oviposition pheromone receptor from recombinant PBP to a heterologous system that expresses a et al. 2006). Recently, it was shown that addition of a 246 J Chem Ecol (2010) 36:245 sensitivity of the mosquito olfactory system. suggest that CquiOBP1 is involved in the detection of to-48 h-old Injected through the intersegmental thorax membranes into 1- Kit (Qiagen). Approximately 100 nl (350 ng) of dsRNA were orientations and purified by using RNeasy MinElute Cleanup product that contained T7 promoter sequences in inverted was synthesized by in vitro transcription from purified PCR was carried out by using EXPRESS SYBR® GreenER™ qPCR Super-Mix Universal (Invitrogen) in a final volume of 25 μl. CquiRpL8 amplification was used as a control of cDNA integrity.

**Methods and Materials**

**CquiOBP1 RNA Interference** Full-length CquiOBP1 dsRNA was synthesized by in vitro transcription from purified PCR product that contained T7 promoter sequences in inverted orientations and purified by using RNeasy MinElute Cleanup Kit (Qiagen). Approximately 100 nl (350 ng) of dsRNA were injected through the intersegmental thorax membranes into 1- to-48 h-old Cx. quinquefasciatus female mosquitoes with a microINJECTOR™ System MINJ-1 (Tritech Research, Los Angeles, CA, USA). dsRNA-injected, water-injected, and non-injected mosquitoes were generated. Individual female heads were dissected in liquid nitrogen 4 d post-injection, RNA from each head was extracted with RNasey Mini Kit (Qiagen), and individual cDNAs were synthesized from 0.1 μg of RNA using 100u SuperScript® II reverse transcriptase (Invitrogen). Real-time quantitative PCR (qPCR) was carried out by using EXPRESS SYBR® GreenER™ qPCR Super-Mix Universal (Invitrogen) in a final volume of 20 μl. Reactions were run with a standard cycling program, 50°C for 2 min, 95°C for 2 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min, on an AB7300 real-time PCR system (Applied Biosystems). Determination of transcripts abundance was based on two independent replicates for each sample. CquiOBP1 expression was normalized to the expression levels of an endogenous control, the ribosomal protein that encodes gene S7 (CquiRpS7). Relative quantification analysis based on the comparative C_t method (ΔΔC_t) was performed using AB7300 system SDS software (Applied Biosystems). Non-injected mosquitoes were used for calibration purposes. Non quantitative PCR was carried out from the same cDNAs by using 2u GoTaq® DNA polymerase (Promega) in a final volume of 25 μl. CquiRpL8 amplification was used as a control of cDNA integrity.

**Electrophysiological Recordings** An excised head of an adult Cx. quinquefasciatus female was mounted on a Syntech EAG platform equipped with micromanipulator-12 and a high-impedance AC/DC preamplifier (Syntech, Germany). Chloridized silver wires in drawn-out glass capillaries filled with 0.1% KCl and 0.5% polyvinylpyrrolidone (PVP) were used for reference and recording electrodes. The recording electrode accommodated the two antennae of the excised head after the tips of the antennae were clipped to provide a better contact. Preparation was bathed in a high humidity air stream flowing at 20 ml/s to which a stimulus pulse of 2 ml/s was added for 500 ms. Any change in antennal deflection induced by the stimuli or control puffs was recorded for 10 s. Indole and 3-methyl indole (skatole) were purchased from Acros (USA) and were 95% pure; nonanal (99%) was from Sigma-Aldrich; racemic 6-acetoxymethylindane (MOP) was a gift from Bedoukian Research Incorporated, USA. Chemicals were dissolved in dichloromethane (DCM), wt/vol, to make a stock solution of 10 μg/μl and decadic dilutions were made. An aliquot (10 μl) of a stimulus was loaded onto a filter paper strip, the solvent was evaporated for 30 s, and the strip was placed in a 5 μl polypropylene syringe from which various volumes were dispensed. Solvent alone served as control. Data presented are from a pool of mosquitoes injected and tested in three different batches on different days. In each session, EAG responses of at least three of RNAi-treated and water-injected mosquitoes were recorded.

**Results and Discussion**

We employed a combination of RT-PCR and real-time quantitative PCR (qPCR) to examine mRNA levels of CquiOBP1 in heads of RNAi (dsRNA-injected) and control (water-injected, non-injected) mosquitoes using CquiRpS7 as a control gene. RT-PCR analysis showed a clear reduction of CquiOBP1 transcript levels in dsRNA-injected mosquitoes, as compared to water-injected and non-injected mosquitoes (Fig. 1a). We then examined by
electroantennogram (EAG) the responses of sham-and RNAi-treated female mosquitoes to oviposition attractants. Silencing the *CquiOBP1* gene clearly affected antennal responses to MOP and indole, a putative oviposition attractant (Millar et al. 1992) (Fig. 1b), but the response to nonanal was not significantly affected. Next, we quantified the reduction of transcripts by qPCR (Fig. 1c), which confirmed the trend observed by a semi-quantitative method (Fig. 1a). dsRNA-injected mosquitoes displayed reduction of *CquiOBP1* transcript levels (average 59.9%) when compared to both water-injected (sham-treated) mosquitoes (average 97.3%) and non-injected controls (normalized to 100%). dsRNA-Injected individuals displayed significant reduction of *CquiOBP1* transcripts (47% to 65%) (Fig. 1c). Furthermore, water-injected and non-injected mosquitoes displayed almost equivalent levels of *CquiOBP1* transcripts, thus demonstrating that RNAi treatment is responsible for the observed reduction of *CquiOBP1* mRNA levels (Fig. 1c). This partial silencing of *CquiOBP1* shown by qPCR analysis demonstrates the feasibility of significantly reducing even highly expressed olfactory genes like OBPs by using the RNAi approach.
Correlation with EAG data (Fig. 1b) also suggests that 
~50% transcripts reduction is enough to generate reduced 
responses to several semiochemicals.

Finally, we compared the responses of sham- and RNAi-
treated female mosquitoes to various doses of these 
ovidiposition-related compounds. EAG responses of RNAi-
treated females to MOP were below the detection limit, but 
the dose required to generate consistent EAG signals with 
water-treated or untreated mosquitoes was high (100 μg). In 
contrast, reduction of CquiOBP1 transcripts led to a 
significantly reduced response to skatole (N=10, P<0.05) 
at all doses tested (Fig. 1d). Likewise, EAG responses to 
indole by RNAi-treated females were significantly lower 
than the responses recorded from water-treated female 
mosquitoes at all doses tested (Fig. 1e). Lastly, we observed 
an apparent trend towards smaller EAG responses to 
nonanal by RNAi-treated compared water-treated female 
mosquitoes, but the differences were not significant 
(Fig. 1f).

The simplest explanation for these findings is that OBPs 
play an important role for the sensitivity of the insect’s 
ofactory system. Although we were not able to completely 
silence CquiOBP1, probably because of the high level of 
transcription, the partial knockdown clearly affected antennal 
response to physiologically relevant compounds. Previously, 
we demonstrated in vitro assays that CquiOBP1 binds 
MOP in a pH-dependent manner, and we showed its 
expression in antennal sensilla sensitive to this oviposition 
attractant (Leal et al. 2008). These RNAi experiments are the 
first evidence in vivo that CquiOBP1 is involved in the 
reception of Culex mosquito oviposition attractants. 
Although it is tempting to speculate that CquiOBP1 is selective 
because responses to nonanal were not significantly different in 
sham- and RNAi-treated mosquitoes (Fig. 1f), the level of 
transcript reduction achieved by our RNAi treatments may 
not be high enough to affect EAG responses of semi-
ochemicals such as nonanal for which the olfactory system 
responds with remarkable sensitivity (Syed and Leal 2009). 
By contrast, the reduced levels of CquiOBP1 transcripts 
affect the responses of compounds with higher thresholds, 
thus allowing us to conclude that CquiOBP1 is indeed 
involved in the detection of oviposition attractants, and that 
high levels of OBPs expression are essential for the 
sensitivity of the insect’s olfactory system.

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Note added in proof: Since this manuscript was accepted for 
publication, the authors became aware of a report by Biessmann et al. 
(describing that RNAi is effective in knocking down accumulation of 
OBP1 transcripts in the antenna of Anopheles gambiae mosquitoes. 
Their results will be reported in the following paper in PLoS ONE: 
Harald Biessmann, Evi Andronopoulou, Max R. Biessmann, Vassilis 
Dourisb, Spiros D. Dimitratos, Elias Eliopoulos, Patrick M. Guerin, 
Kostas Iatrou, Robin W. Justice, Thomas Kröber, Osvaldo Marinotti, 
Panagiota Tsitoura, Daniel F. Woods, Marika F. Walter. The Anopheles 
gambiae Odorant Binding Protein 1 (AgamOBP1) mediates indole 
recognition in the antennae of female mosquitoes. IN PRESS.

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