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Chitosan/DsiRNA nanoparticle targeting identifies AgCad1 cadherin in *Anopheles gambiae* larvae as an *in vivo* receptor of Cry11Ba toxin of *Bacillus thuringiensis* subsp. *jegathesan*

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ABSTRACT

The Cry11Ba protein of *Bacillus thuringiensis* subsp. *jegathesan* crystals has uniquely high toxicity against a spectrum of mosquito species. The high potency of Cry11Ba against *Anopheles gambiae* is caused by recognition of multiple midgut proteins including glycosyl phosphatidylinositol-anchored alkaline phosphatase AgALP1, aminopeptidase AgAPN2, α-amylase AgAmy1 and α-glucosidase Agm3 that bind Cry11Ba with high affinity and function as putative receptors. The cadherin AgCad2 in *An. gambiae* larvae also binds Cry11Ba with high affinity ($K_d = 12$ nM) and is considered a putative receptor, while cadherin AgCad1 bound Cry11Ba with low affinity ($K_d = 766$ nM), a property not supportive for a Cry11Ba receptor role. Here, we show the *in vivo* involvement of AgCad1 in Cry11Ba toxicity in *An. gambiae* larvae using chitosan/DsiRNA nanoparticles to inhibit AgCad expression in larvae. Cry11Ba was significantly less toxic to AgCad1-silenced larvae than to control larvae. Because AgCad1 was co-suppressed by AgCad2 DsRNAi, the involvement of AgCad2 in Cry11Ba toxicity could not be ascertained. The ratio of AgCad1:AgCad2 transcript level is 36:1 for gut tissue in 4th instar larvae. Silencing AgCad expression had no effect on transcript levels of other binding receptors of Cry11Ba. We conclude that AgCad1 and possibly AgCad2 in *An. gambiae* larvae are functional receptors of Cry11Ba toxin *in vivo*.

1. Introduction

Protozoa and viruses vectored by mosquitoes cause severe human diseases including malaria, Dengue, West Nile Virus and chikungunya. Disease transmission can be disrupted by strategies that target the adult stage with chemical pesticides, or by targeting the larval stage by treating breeding sites with either insect growth regulators or the entomopathogenic bacterium *Bacillus thuringiensis* (Bt) subsp. *israelensis* (Bti). When applied to mosquito breeding sites, formulations of Bti spores and parasporal crystals are highly effective against larval stages of *Aedes* and *Anopheles* but less effective against *Culex* species (Poncet et al., 1995).

The mosquitocidal activity of Bti involves its proteinaceous crystals which are composed of Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1Aa and Cyt2Ba proteins (Ben-Dov, 2014; Berry et al., 2002). Concerns regarding the potential for mosquito resistance and the desire to have greater activity against certain species led to the discovery of *Bt* subsp. *jegathesan* (Btjeg) (Selena et al., 1995). The crystals of Btjeg have eight insecticidal proteins (Sun et al., 2013), of which Cry11Ba has been studied in the most detail (Delecluse et al., 1995) as Cry11Ba is the single most active Cry protein across the three major genera of disease-vectoring mosquitoes.

Midgut proteins in the midgut brush border of larvae bind Cry toxins and mediate events culminating in larval death [for recent reviews refer to (Adang et al., 2014; Pardo-López et al., 2013; Vachon et al., 2012). Alkaline phosphatases and aminopeptidases are midgut receptors of Bti Cry4Ba and Cry11Aa, and Btjeg Cry11Ba toxins in *Aedes aegypti* (Fernandez et al., 2006; Jimenez et al., 2012; Likitvivatanavong et al., 2011). An aminopeptidase binds Cry11Ba in

Abbreviations: Bt, *Bacillus thuringiensis*; BSA, bovine serum albumin; BBMV, brush border membrane vesicles; AgALP1, *Anopheles gambiae* alkaline phosphatase; AgAPN2, *Anopheles gambiae* aminopeptidase N; AgCad1, *Anopheles gambiae* cadherin 1; AgCad2, *Anopheles gambiae* cadherin 2; Agm3, *Anopheles gambiae* glucosidase; CR, cadherin repeat; DsiRNA, Dicer substrate short interfering RNA; RNAi, RNA interference; RT-qPCR, Real Time quantitative polymerase chain reaction; PCR, polymerase chain reaction; SP, signal peptide; TM, transmembrane.

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Anopheles albimanus (Abdullah et al., 2006) and receptor function was established for a related aminopeptidase in *An. gambiae* larvae (Zhang et al., 2008). An α-amylase is a receptor of Cry4Ba and Cry11Aa in *An. albimanus* and for Cry11Ba in *An. gambiae* (Fernandez-Luna et al., 2010; Zhang et al., 2013). Cadherin was originally identified as a Cry1Ab receptor on the brush border membrane of *Manduca sexta* (Vadlamudi et al., 1993). Cadherin was found not only to be involved in toxin binding, but the cadherin repeats (CR) showed a synergistic effect to Cry1A toxin in lepidopterans (Abdullah et al., 2009; Chen et al., 2007; Peng et al., 2010) and to Cry4Ba toxin in dipterans (Hua et al., 2008; Park et al., 2009). In *Ae. aegypti*, cadherin was identified as a binding receptor of Cry11Aa and Cry11Ba (Chen et al., 2009a; Lee et al., 2014). As for *An. gambiae*, cadherin AgCad2, which shares only 14% identity at the amino acid level with the Cry4Ba-binding AgCad1, bound Cry11Ba with high affinity and cadherin repeats (CR) peptides showed an inhibitory effect to Cry11Ba toxicity against *An. gambiae* larvae (Hua et al., 2013). *Aedes* cadherin mediates *in vivo* toxicity of Cry11Aa to *Ae. aegypti* larvae, as evidenced when cadherin-silenced *Ae. aegypti* larvae became less susceptible to Cry11Aa (Lee et al., 2014; Rodriguez-Almazan et al., 2012).

RNAi has been employed as an effective tool to investigate the *in vivo* roles that receptors have for Cry toxin toxicity. Silencing of GPI-anchored APN isoforms (APN2783, APN5808, and APN2778) led to different levels of tolerance to Cry4Ba toxicity in *Ae. aegypti* larvae (Saengwiman et al., 2011). One of the APN isoforms (APN2778) was previously reported as a binding receptor of Cry11Aa (Chen et al., 2009b). Another GPI-anchored protein, ALP1, was silenced in *Ae. aegypti* larvae, resulting in a tolerant response against Cry4Ba and Cry11Aa (Jimenez et al., 2012). Moreover, silencing of heat shock protein, ATP synthase beta subunit or actin caused two to four fold increased susceptibility to Cry11Aa in *Ae. aegypti* larvae (Cancino-Rodezno et al., 2012).

While the success of RNA inhibition as a functional approach in *Ae. aegypti* larvae is well-established, it is less so in *An. gambiae* larvae. Zhang et al. (2010b) reported successful gene silencing using chitosan-entrapped dsRNA nanoparticles in *An. gambiae*. The recent success of Mysore et al. using chitosan-DsiRNA nanoparticles to achieve successful gene silencing in *Ae. aegypti* (Mysore et al., 2014, 2013), prompted our use of chitosan/DsiRNA nanoparticle-mediated RNAi to investigate the *in vivo* roles of two cadherins, AgCad1 and AgCad2, for Cry11Ba toxicity in *An. gambiae* larvae. We expected AgCad2, but not AgCad1, to have an *in vivo* role in Cry11Ba toxicity to larvae, but our observed results did not support this hypothesis. Rather, our results support the role of AgCad1, and possibly AgCad2, as mediating Cry11Ba toxicity in *An. gambiae* larvae.

2. Materials and methods

2.1. Insects

An. gambiae (CDC G3 strain) were maintained at 27 °C with a light-dark photoperiod of 16h: 8h as described (Hua et al., 2008). Larvae of the appropriate age were collected and used for RNAi and other experiments.

2.2. Purification of Cry11Ba protoxin

The Bt strain 407, harboring plasmid pJEG80.1 encoding Cry11Ba (Delécluse et al., 1995) was grown in sporulation medium with erythromycin as described previously (Hua et al., 2008). Spores and crystals were harvested by centrifugation and washed according to Hua et al. (2008). Crystals were purified from spores by centrifugation through a 30–60% (w/v) NaBr step gradient at 47,000 *g* for 2 h. Purified crystals were washed twice with deionized H_2O and then dissolved in 25 ml 100 mM 3-(cyclohexylamino) propanesulfonic acid (CAPS), pH10.6, supplemented with 0.05% β-mercaptoethanol overnight at 5 °C to release protoxin. The solution was clarified by centrifugation at 10,000 *g* for 10 min and filtration through a 0.45 μM BA85 membrane filter (Whatman). The solution was dialyzed overnight at 5 °C against 20 mM sodium carbonate pH 9.6. Protoxin was bioassayed against early 4th instar larvae to calculate an LC_{60} dosage for use in RNAi experiments (described below).

2.3. Preparation of chitosan/DsiRNA mediated nanoparticles for mosquito RNAi

Cadherin knockdown was performed by orally feeding chitosan/DsiRNA nanoparticles to *An. gambiae* larvae. Two DsiRNAs, DsiRNA-1 and DsiRNA-2 corresponding to different regions of each cadherin gene AgCad1 or AgCad2 were designed with the Integrated DNA Technologies (IDT; Coralville, IA, USA) website at http://www.idtdna.com/site. Scrambled sequences to AgCad2 (DsiRNAs) lacking a target to any known *An. gambiae* gene were determined by blast search against the NCBI database and used as a negative control. Two of the DsiRNAs of scrambled sequence to AgCad2 were selected using a software tool available on Genscript: https://www.genscript.com/ssl-bin/app/scramble. Food pellets mixed with chitosan without DsiRNAs was used as another control. All DsiRNAs were chemically synthesized by IDT Company (Coralville, IA, USA), with sense and antisense sequences shown in Table 1. The method of preparation of chitosan/DsiRNA nanoparticles was adapted from Mysore et al. (2013) and Zhang et al. (2010b). Two DsiRNA constructs targeting the same cadherin were combined to make one chitosan/DsiRNAs nanoparticle pellet. Each pellet containing 32 μg of each of the two DsiRNAs was dissolved in 50 μl 50 mM Na_2SO_4. The combined DsiRNA were incorporated with 100 μl 0.04% chitosan. After centrifuging at 13,000 g, the pellet was mixed with 6 mg of food (Tetra Min, tropical flakes chopped with a bladed coffee grinder) with dry yeast (2:1 ratio of food: dry yeast) and coated in 15 μl 2% agarose (Fisher Scientific) gel. The embedded pellet was divided into four pieces, and a piece was fed to 65 larvae every 4 h continuously for 3 days for 18 feedings in total. Larvae were maintained in 50 ml distilled water in a Ziploc^R brand square small container (24 oz.), and collected 4 h after the final feeding for total RNA extraction and bioassay.

2.4. RNA extraction and cDNA synthesis

Total RNA extracted from larvae was used to synthesize cDNA for testing the transcript levels of AgCad1 and AgCad2. Eight larvae in three biological replicates were collected from each of the two separated feeding biological replicates and soaked in 200 μl of TRIzol reagent (Ambion) in a microfuge tube. Samples were homogenized using a cordless motor-driven pellet pestle (Grainger)

Table 1
Synthetic DsiRNAs used for RNAi.

AgCad1-DsiRNA-a sense: CUACGAGGUGCAUCAGCGUAGCAGT
AgCad1-DsiRNA-a antisense: CUGAUGCUCCACGUAGUCGCAUCGUCA
AgCad1-DsiRNA-b sense: CGAUAAUGCUCCUUACUUUGACAAC
AgCad1-DsiRNA-b antisense: UUGCUAUUACGAGGAAUGAAACUCUUG
AgCad2-DsiRNA-a sense: CGAGGAUGAUCUAUUCUCUUUCAAC
AgCad2-DsiRNA-a antisense: ACGCUCCUACUAGAUAAGAGAAAGUUG
AgCad2-DsiRNA-b sense: GGUCAACUGAGCUUCGACAUUGUGG
AgCad2-DsiRNA-b antisense: UGCCAGUUGACUCGAAGCUGUAACACC
Scrambled Control DsiRNA sense: GCUCAUAUUCUAAGAUUGACCUCUG
Scrambled Control DsiRNA antisense: CGAGUAUAAGAUUCUAACUGGAGAC