SHORT REPORT

Silencing of carbonic anhydrase in an *Anopheles gambiae* larval cell line, Ag55

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ABSTRACT

RNAi has been used extensively to down-regulate proteins in adult mosquitoes; however, it is not well adapted for use in larvae. Larval mosquitoes can generate a pH as high as 10.5 in the anterior region of their midgut. The mechanisms responsible for the generation and maintenance of this pH are not entirely understood, but members of the carbonic anhydrase (CA) family of enzymes have been implicated. Here we use an *An. gambiae* larval cell line, Ag55 cells, to demonstrate that application of full-length double-stranded RNA specific to one CA, AgCA9, is sufficient to silence AgCA9 mRNA and down-regulate the corresponding protein. This is a first step towards determining the role(s) of these enzymes in pH regulation.

KEYWORDS: Carbonic anhydrase, Ag55, larval cell, *Anopheles gambiae*, RNAi, gene silencing

INTRODUCTION

RNA interference (RNAi) is a powerful tool for manipulating mRNA levels and has been used in a variety of systems (for example, Elbashir et al, 2001; Zhao et al, 2007). One particular group of organisms in which RNAi is used extensively is arthropods, and the technique has been demonstrated in many arthropod species including mosquitoes (Lu et al, 2004). However, although RNAi has been adapted for use in adult mosquitoes (Blandin et al, 2002) it is not well established for mosquito larvae. Therefore, the demonstration of successful RNAi in a larval mosquito cell line will be an important step towards manipulating mRNA levels *in vivo*. The manipulation of larval gene expression is a crucial step in understanding the mechanisms responsible for their survival. Of particular interest to our laboratory is the regulation of genes involved in larval pH regulation.

Mosquito larvae generate a highly alkaline pH (~10.5) in a restricted area of their alimentary canal (AC), the anterior midgut (AMG) (Dadd, 1975), despite the absence of morphological barriers between the AMG and adjacent, more neutral regions of the AC. The alkaline environment is thought to aid in digestion and is crucial for larval survival (Corena et al, 2004). However, the molecular mechanisms responsible for the generation and maintenance of the pH gradient are not fully understood. A better understanding of these mechanisms could pave way for the discovery of novel targets for new and improved larvacides.

One group of proteins with a known role in AMG alkalization is the carbonic anhydrase (CA) family (del Pilar Corena et al, 2002). There are a predicted twelve genes belonging to the *Anopheles gambiae* CA family (www.ensembl.org), six of which have been cloned by members of our laboratory (Smith et al, 2007). Our laboratory has detected CA within the epithelial cells of the mosquito larval AC in both *An. gambiae* and *Aedes aegypti* using various methods (del Pilar Corena et al, 2002; Corena et al, 2004; Seron et al, 2004; Smith et al,

2007; Neira Oviedo et al, 2008). Additionally, we established that CA is necessary for mosquito larval alkalization and survival (Corena et al, 2004).

Currently, the specific roles of each CA member are unknown. A first step in determining if any one CA or combination of CAs is responsible for pH regulation is to silence them either individually or in concert. In order to determine whether CAs can be manipulated by RNAi, we used an *An. gambiae* larval cell line, Ag55, to demonstrate CA silencing. Here, we report the mRNA down-regulation of an abundant AC CA, AgCA9 (GenBank accession number DQ518576) in Ag55 cells and the downstream knockdown of the protein product.

MATERIALS AND METHODS

Polymerase chain reactions

Nucleotide sequence for primers and thermal cycling parameters used in the various polymerase chain reactions are given in Table 1.

Ag55 cells

Ag55 cell line (Pudney et al, 1979) was a gift from Kimberly Keene (Colorado State University). The cells were grown in 75 cm^2 flasks (Fisher Scientific, Pittsburgh, PA) at 28°C in Leibovitz's L-15 media (Sigma-Aldrich, St Louis, MO) supplemented with 10% (v/v) fetal bovine serum (FBS, Atlantic Biologicals, Norcross, GA), 1% (v/v) Penicillin-Streptomycin solution (10,000U/ml and 10mg/ml, respectively) (Sigma-Aldrich). The cell culture medium was changed every other day.

Detection of AgCAs

RNA was extracted from 10 x 10^6 Ag55 cells or 20-30 whole *An. gambiae* fourth-instar larvae using TriZol Reagent (Molecular Research Center, Inc, Cincinnati, OH). Genomic contamination was removed using the TURBO DNA-*free*™ kit (Ambion, Austin, TX). RNA was reverse transcribed into cDNA using SuperScript® III Reverse Transcriptase (Invitrogen, Carlsbad, CA). All procedures were conducted according to manufacturer's instructions.

cDNA from Ag55 cells or whole larvae was used as a template for PCR reactions with primers specific to one of the twelve predicted *An. gambiae* CA genes. Primers were designed to amplify whole mRNA, in the case of those fully cloned and sequenced genes, or a portion of the mRNA predicted by the *An. gambiae* genome (www.ensembl.org; February 2006 update). The PCR product was gel extracted (Qiagen Gel-extraction kit, Valencia, CA), ligated into the pCR4-TOPO vector (Invitrogen), and sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing Kit (PE Biosystems, Foster City, CA). The reaction products were analyzed in an ABI Prism 310 Genetic Analyzer.

RNA production

RNA was produced by *in vitro* transcription in three steps: creation of double-stranded (ds)DNA flanked by the T7 promoter sequence on either the 5' or 3' end, transcription of sense and antisense strands into single-stranded RNA (ssRNA), and finally, annealing of the two single strands into dsRNA. Primers were designed to amplify either AgCA9 or eGFP DNA and to add a T7 promoter sequence at either the 5' or 3' end. eGFP cDNA was a gift from Lyric Bartholomay (University of Wisconsin). The PCR products were purified using the Qiagen PCR purification kit (Qiagen) according to manufacturer's instructions.

Sense (from dsDNA with 5' T7) and antisense (from dsDNA with 3' T7) RNA were transcribed from the above dsDNA using the MEGAscript T7 transcription kit (Ambion) and treated with TURBO DNase (Ambion) according to manufacturer's instructions. Each ssRNA strand was then adjusted to equal concentrations for annealing. Equal volumes of each strand were combined and incubated in a 95°C water bath for five minutes; the heat was removed and the water bath (with tubes in it) was allowed to cool to room temperature overnight.

RNAi experiments

Ag55 cells were grown to 70% confluency in six well plates for experimental (AgCA9 dsRNA treated) and two control groups (eGFP dsRNA treated and untreated). The untreated group did not receive dsRNA, but was subjected to the same conditions as the other two groups. To treat the cells, media was replaced with 800µl serum-free medium plus 36.0µg dsRNA. The plate was rocked at room temperature for 30min and then 800µl medium plus 20% FBS was added. Cells were incubated at 28ºC and harvested at 24, 48, 72 or 96 hr post-treatment; after 96hr, the cells began to overgrow and die. To harvest, the cells were washed twice with 2.0 ml serum-free medium and resuspended in 0.4 ml Trizol Reagent to extract RNA and protein according to manufacturer's instructions.

Quantitative PCR (q-PCR)

RNA from each set of cells was treated with DNase and reverse transcribed as described previously. In order to detect AgCAs, Q-PCR primers were designed to amplify ~50 bp fragments of both AgCA9 3' UTR or an 18s ribosomal RNA control using ABI primer express software. The reactions were prepared and run using SYBR Green Master Mix (ABI) with 300µM of each AgCA9 primer or 100µM of each 18s primer in a 96 well plate; each reaction was run in triplicate. Q-PCR was performed using Applied Biosystems (ABI) 7000 Sequence Detection system and data were analyzed using the relative expression method described in Pfaffl (2001).

Northern blotting

To generate a radioactive northern probe, a pCR4-TOPO vector containing full-length AgCA9 was linearized by restriction enzyme digest with either *Pst*I or *Not*I. This template was then reverse transcribed and radiolabeled with ^{32}P-labeled dUTP (800Ci/mmol, 20mCi/ml; GE Healthcare Bio-Sciences Corp, Piscataway, NJ) using the Maxiscript kit (Ambion). The NorthernMax Kit protocol (Ambion) was used to run and analyze the northern blot. All procedures were conducted according to manufacturer's instructions. The following amounts of RNA were loaded for each time point: 48hr - 10µg, 72hr - 6.3µg, and 96hr - 8.0µg. Less than 1.0µg RNA was extracted from 24hr time point cells, and could not be visualized on the northern blot.

Table 1. Primers used for PCR. T7_AgCA9 and T7_GFP refer to primers that generated products with a 5' T7 promoter sequence. AgCA9_T7 and GFP_T7 refer to primers that generated products with a 3' T7 promoter sequence.

CA	ENSEMBL ID	Forward primer	Reverse primer	Length (bp)	Annealing Temp (°C)	cycles
RT-PCR primers						
AgCA1	AGAP002360	ATGAGTGCGCTGTGTTTG	ATCCAGTTGACCGTCTCC	800	57	35
AgCA-RP2	AGAP000715	CGAGCCATGTCAGTCAT	CTATGGAAGCGAATGGAAC	1032	55	35
AgCA3	AGAP000401	CACTACGTGGGCCACTGGGAC	CTAAAGTTTGGCGCGCCG	592	55	40
AgCA4	AGAP007550	AATAGCTTCAACCACCAAACC	GTGTCAGTGGGTGTCCTTC	913	57	35
AgCA-RP5	AGAP000688	CCGACAGTCACCCGTCA	GGACCACCCTGCATTAAC	657	63	40
AgCA6	AGAP003289	ATGCAGTTGTCGATTGGATC	CTAGTACACCCAGTCCCATTTG	957	60	35
AgCA7	AGAP002359	AGTTCACTTACCGCATCTCG	TGGAACTGATCGAACAGGTAC	904	57	40
AgCA8	AGAP010844	TCGTATGGTCACTGGCAC	GACACTGCACTTCGTCCTC	930	58	35
AgCA9	AGAP010052	ATGTCTGTCACTTGGGGATACAC	TTAGTAGCTATCGACTTCACGCAG	831	60	35
AgCA10	AGAP004895	ATGAAAAGTTTCACTTTATTGCTCTG	TCAGTGGTAACTCAATCTGGATG	960	60	35
AgCA11	AGAP005066	ATGGCATCAAAAACAACAAAGAAC	TTACAGCTTCGAAAGCACAACGG	774	63	40
AgCAb	AGAP002992	ATGGAGCGTATATTGCGAG	TCACGAATAGTATCGCCGTAC	768	60	35
T7 PCR primers						
T7_AgCA9		TAATACGACTCACTATAGGGATGTCTGTCACTTGGGGATACAC	TTAGTAGCTATCGACTTCACGCAG	871	60	30
AgCA9_T7		ATGTCTGTCACTTGGGGATACAC	TAATACGACTCACTATAGGGTTAGTAGCTATCGACTTCACGCAG	871	60	30
T7_GFP		TAATACGACTCACTATAGGGGAGGTGAAGTTCGAGGGC	TCCATGCCGAGAGTGATC	411	55	30
GFP_T7		GAGGTGAAGTTCGAGGGC	TAATACGACTCACTATAGGGTCCATGCCGAGAGTGATC	411	55	30
Q-PCR primers						
AgCA9		CAACGGTAGGAGTATGGTCGATCT	ACGTACCGCGCTTTGCA			
18s		GCGACCTCGTCGGTCAAG	AGAGTTCCCGGGCACCAT			

Western blotting

Chicken antibodies were generated by Aves Labs, Inc (Tigard, OR) against the BSA-conjugated peptide: CZELGNRQLREVDSY and were used as purified IgY (Smith et al, 2007). To detect AgCA9 from each set of Ag55 cells (those treated with AgCA9 dsRNA or eGFP dsRNA, or those left untreated for 24, 48, 72 and 96 hr), western blotting was performed as described in Smith et al (2007). In addition to the total protein stain a second protein, which cross-reacted with the antibody, distinguishable by its slightly higher molecular weight, was used to support equal loading of each sample.

RESULTS AND DISCUSSION

Expression of AgCAs

The Ag55 cell line is an *An. gambiae* neonatal first-instar larval cell line which has been used by others for successful RNAi silencing (Konet et al, 2007). PCR was used to determine which CA genes were expressed in Ag55 cells, using whole larvae as a control template (Figure 1). All but AgCA1 and AgCA7 were detectable in the whole larvae controls using the indicated primers. It is possible that these CAs were expressed at a level too low to be detected using our methods. However, the expression

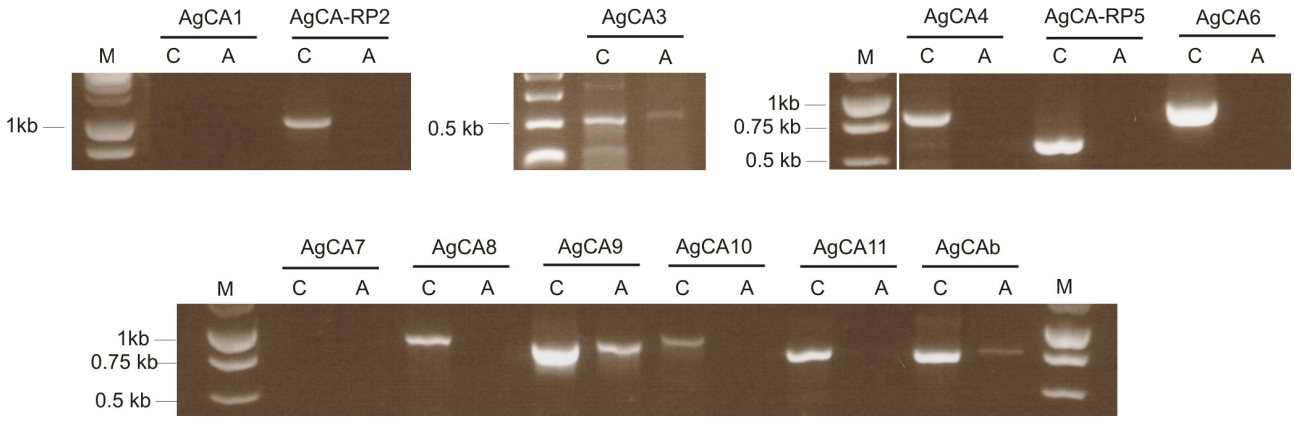

Figure 1. PCR detection of each *An. gambiae* α–CA (AgCA1-11) and the one β–CA (AgCAb) in Ag55 cells. Ag55 cDNA (indicated by an "A" above the lane) or whole *An. gambiae* fourth-instar larvae cDNA (indicated by a "C" above the lane) was used as a template. See table 1 for primer sequences and expected length of products. M: 1 kb DNA ladder® (invitrogen).

of many CA genes is known to be highly-variable between larval and adult specimens (Dissanayake et al, 2006). Thus, it is also possible that these CAs might not be expressed at this particular developmental stage. Ag55 cells expressed AgCA3, AgCA9 and AgCAb. AgCA9 is a good candidate for a role in larval pH regulation due to the high-level mRNA expression in the gastric caeca and ectoperitrophic space (Smith et al, 2007). Therefore we chose to test whether this CA could be silenced in Ag55 cells using RNAi.

Reduction of AgCA9 mRNA and protein levels by using dsRNA

Ag55 cells were treated with full length AgCA9 dsRNA, eGFP dsRNA or were left untreated for 24, 48, 72 or 96 hr. Treatment with AgCA9 dsRNA resulted in a statistically significant (P-value <0.05) knock-down of AgCA9 mRNA compared to the eGFP dsRNA or untreated cells as determined by q-PCR analyses (Figure 2A). This knock-down became evident after 24hr and persisted for at least 96hr, exhibiting 88% reduction in AgCA9 mRNA level at 96hr time-point. These time-point data are consistent with previous reports, which suggested RNAi silencing to occur in as little as 24hr (Blitzer et al, 2005) and to last for at least ten days (Keene et al, 2004).

Northern blot analysis performed in parallel with q-PCR supported a considerable knock-down of AgCA9 mRNA in cells treated with AgCA9 dsRNA (Figure 2B). A distinct AgCA9 mRNA band was detected at the expected size (~1.5 kb), including un-translated regions, in the control lanes, which was absent from the cells treated with AgCA9 dsRNA. These data, in conjunction with q-PCR results, demonstrate that introduction of dsRNA is sufficient to silence AgCA9 mRNA in Ag55 cells.

When using RNAi to investigate protein function, the protein half-life must be considered. If the protein is stable, for example, with a half-life that exceeds the length of the experiment, protein levels may remain unchanged even if mRNA expression is decreased. However, there are no clear data to indicate the half-life of AgCA9 protein. We therefore, tested by western blotting whether we could reduce the AgCA9 levels parallel with the mRNA levels. Western blot analysis demonstrated that AgCA9 protein was indeed considerably down-regulated in cells treated with AgCA9 dsRNA compared with the controls (Figure 3). These results indicate that AgCA9 protein is capable of being manipulated by RNAi and suggests that this technique has the potential for *in vivo* silencing of CAs. By down-regulating CA protein in mosquito larvae, pH changes can be assessed and relative contributions of each CA to AMG pH regulation can be determined.

Several RNAi-based methods have been applied to silence genes in adult mosquitoes: These include direct injection of dsRNA (Boisson et al, 2006; Roy et al, 2007; Hansen et al, 2007), introduction of a transgene to deliver hairpin RNA (Franz et al, 2006), and infection with a virus that produces the dsRNA of interest (Adelman et al, 2001). The most straightforward approach to RNAi in mosquitoes appears to be direct injection of dsRNA into the hemocoel. Because the AC is only one cell thick, and mosquitoes have an open circulatory system, therefore, in principle, injected dsRNA has access to virtually every AC cell. Down-regulation of AgCA9 has proved difficult in our hands: We have attempted this by injecting full-length dsRNA, 300 bp dsRNA, or siRNAs into the hemolymph of *An. gambiae* for various time-points and at various concentrations with little success. Despite much work dedicated to larval RNAi on the part of these authors and others, to date there is only one report of successful RNAi in mosquito larvae via injection of dsRNA (Blitzer et al, 2005), and one report using transgenic larvae (Brown et al, 2003).

The apparent difference in RNAi susceptibility between mosquito adults and larvae may be due to the inability of larval cells to uptake dsRNA in a systemic manner, as suggested for *Drosophila melanogaster* (Miller et al, 2008). Our work indicates that, as in *D. melanogaster*,

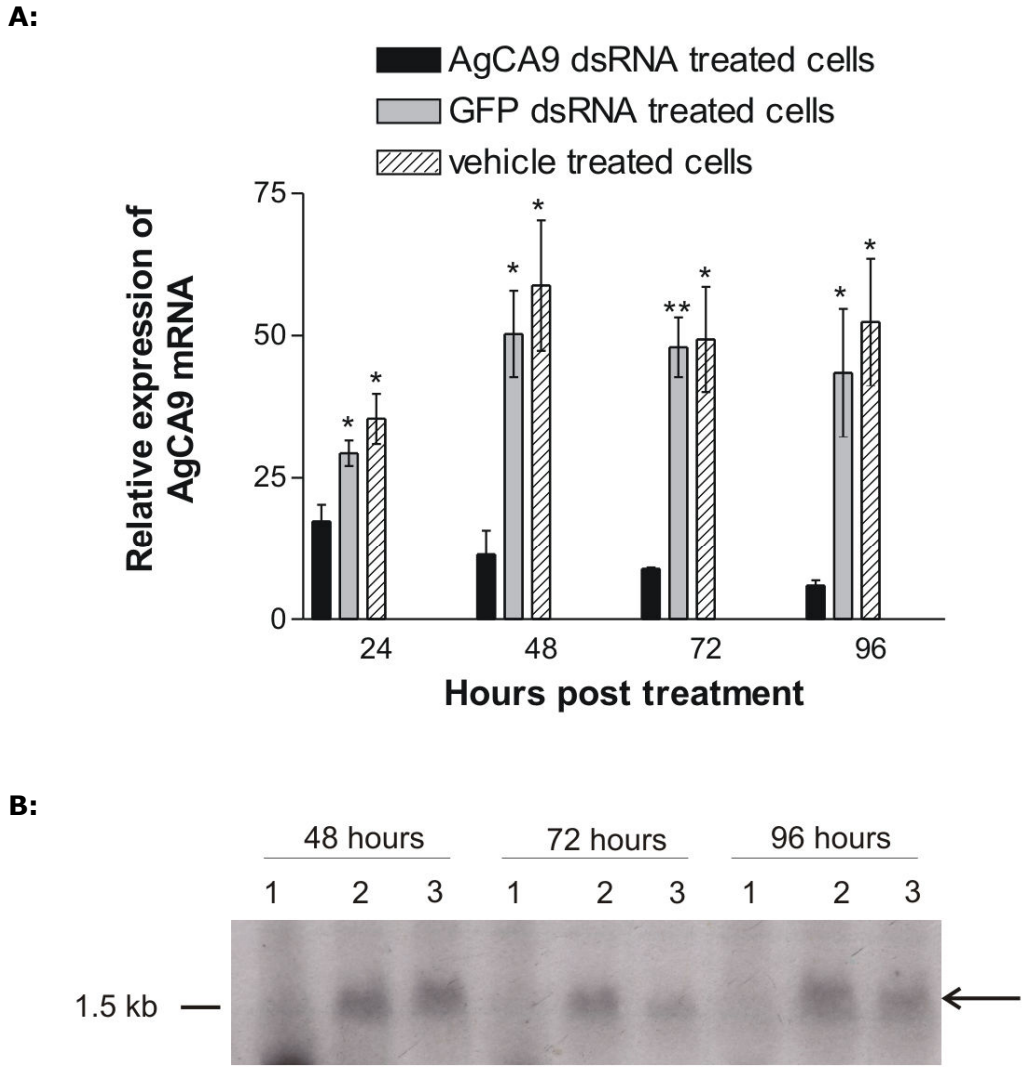

Figure 2. Analyses of AgCA9 mRNA expression. Quantitative PCR (**A**) and northern analysis (**B**) were used to determine endogenous AgCA9 mRNA levels in Ag55 cells which had been treated with either AgCA9 dsRNA, eGFP dsRNA, or left untreated for 24, 48, 72, or 96 hours. For northern analysis- lanes 1: AgCA9 dsRNA-treated cells; lanes 2: eGFP dsRNA-treated cells; lanes 3: untreated cells. *: P-Value < 0.05, **: P-Value < 0.005.

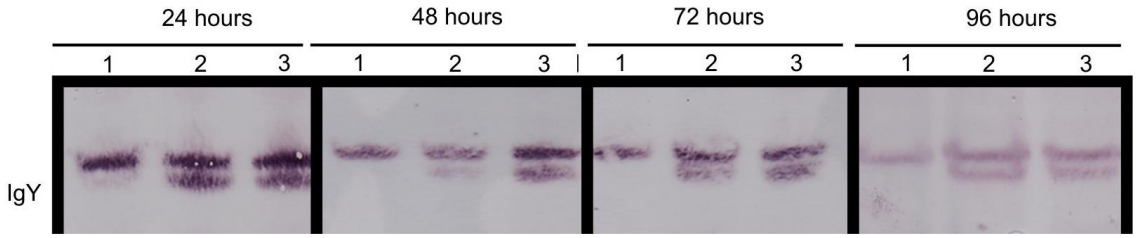

Figure 3. Detection of AgCA9 protein. Western blotting was performed using protein from Ag55 cells which had been treated with either AgCA9 dsRNA (lanes 1), eGFP dsRNA (lanes 2), or left untreated (lanes 3) for 24, 48, 72, or 96 hours. The lower band is AgCA9 protein.

larval mosquito cells possess the RNAi machinery. However, for *in vivo* success delivery of intracellular dsRNA may be the critical step. We have demonstrated that the half-life of AgCA9 protein is likely to be short enough to facilitate RNAi-mediated down-regulation in *An. gambie* larvae. Further work in *An. gambiae* larvae, and possible generation of a transgenic line, will yield a greater understanding of this important family of genes in the crucial process of pH regulation.

CONCLUSIONS

In conclusion we demonstrate that AgCA9 dsRNA can down-regulate mRNA and protein levels in cultured larval cells. Furthermore, protein down-regulation occurs within 24 hours and lasts for at least 96 hours.

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COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

AC; Alimentary canal
AMG; Anterior midgut
CA; Carbonic anhydrase
FBS ; Fetal bovine serum
q-PCR ; Quantitative PCR

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