FEBS Letters 580 (2006) 1988–1992

Gene silencing in mosquito salivary glands by RNAi

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Received 7 February 2006; revised 27 February 2006; accepted 27 February 2006

Available online 6 March 2006

Edited by Gianni Cesareni

Abstract **Salivary glands are the ultimate site of development in the insect of mosquito born pathogens such as *Plasmodium*. Mosquito salivary glands also secrete components involved in anti-haemostatic activities and allergic reactions. We investigated the feasibility of RNAi as a tool for functional analysis of genes expressed in *Anopheles gambiae* salivary glands. We show that specific gene silencing in salivary glands requires the use of large amounts of dsRNA, condition that differs from those for efficient RNAi in other mosquito tissues. Using this protocol, we demonstrated the role of *AgApy*, which encodes an apyrase, in the probing behaviour of *An. gambiae*.**

Keywords: Apyrase; RNAi; Salivary gland; *Anopheles*; *Plasmodium*

1. Introduction

Anopheles mosquitoes are the only mosquitoes able to transmit the *Plasmodium* parasites that cause Malaria in humans. During the last decade numerous studies have been conducted to analyse the genetic basis of *Anopheles* susceptibility to *Plasmodium*. This is now greatly facilitated by the completion of the *Anopheles gambiae* genome sequence and the demonstration that *Anopheles* genes could be silenced by RNAi. Indeed, RNAi has been achieved by injection of dsRNA to the target gene into the haemolymph of the insect [1]. This methodology has proved successful for silencing midgut and haemocyte-specific genes [1–3] and therefore could be used for investigating the role of mosquito genes on the development of *Plasmodium*. Another cell type, in addition to midgut cells and haemocytes, encountered by *Plasmodium* is acinar cells in the mosquito salivary glands. So far, no report has demonstrated the usefulness of RNAi for silencing genes expressed in the mosquito salivary glands. Understanding the role of salivary gland components in the maturation and transmission of *Plasmodium* may give clues to limit the impact of the disease.

Here we report that the RNAi conditions for efficient gene silencing in mosquito midgut cells and haemocytes are not sufficient for silencing gene expression in salivary glands, probably due to a poor permeability of this tissue to dsRNA. However, we succeeded in obtaining reliable and specific gene silencing in salivary glands by using large amounts of injected dsRNA and applied this protocol to analyse the function of *AgApy*, a salivary gland-specific gene encoding a putative apyrase [4].

2. Results and discussion

2.1. *Anopheles* salivary glands are refractory to conventional RNAi

Silencing of gene expression in mosquito midgut cells and haemocytes can be obtained by injection into the insect haemolymph of ~140 ng of dsRNA to the target gene [1,3]. Using this procedure, we targeted the *gfp* gene in a transgenic GFP *An. gambiae* line that strongly expresses GFP in the salivary glands [5]. In this line, GFP is also expressed in at least two anterior midgut cells, the muscle cells surrounding the midgut and the ovaries. The GFP fluorescence was monitored over 12 days post-injection (pi) and compared to the fluorescence pattern in water-injected mosquitoes. Fluorescence in salivary glands was not modified by injection of ds-*gfp* whereas fluorescence disappeared from the two anterior midgut cells and decreased in ovaries and midgut muscle cells (Fig. 1A). This suggested that absence of a decrease of fluorescence was presumably not due to the stability of the GFP protein, but rather to a lack of efficiency of the current RNAi protocol for silencing genes in salivary glands. Indeed, using the same protocol and monitoring gene expression by quantitative RT-PCR, we failed to decrease expression of two salivary gland-specific genes, *AgApy* and *SG1L3* [6,7], whereas silencing of *cpbAg1* [8] a midgut-specific gene was successful (S1).

To investigate further the lack of silencing effect in *Anopheles* salivary glands, we first verified that the major genes involved in the RNAi process were indeed expressed in this tissue. As shown in Fig. 2, *dicer1*, *dicer2*, *ago2* and *ago3*, the latter three being reported as essential for RNAi in *An. gambiae* [9], were expressed in all tissues examined, although their levels of expression were reduced in salivary glands. Since *Anopheles* are known to be poor transmitters of arboviruses, presumably due to a limited permeability of their salivary glands to these viruses, we investigated next whether salivary glands could take up dsRNA molecules. AlexaFluor 555-labelled siRNA (si-*Alexa*) or non-labelled siRNA (si-*gfp*) were injected into the insect haemolymph and fluorescence was recorded over 36 h pi. Fluorescence was detected in midguts and pericardial

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0014-5793/$32.00
doi:10.1016/j.febslet.2006.02.069

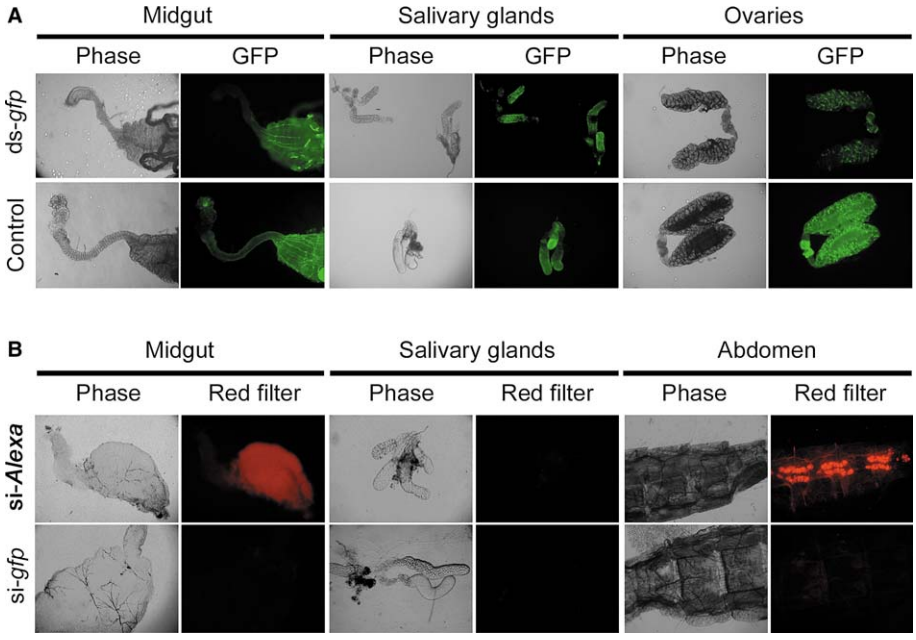

Fig. 1. Effect of in vivo injection of ds-*gfp* and fluorescent si-RNA in a GFP transgenic *An. gambiae* line. (A) Mosquitoes were injected with 140 ng ds-*gfp* or water. Fluorescence was monitored in midguts, salivary glands and ovaries of ds-*gfp*-injected (upper panel) and water-injected mosquitoes (lower panel), 4 days pi. (B) Mosquitoes were injected with 2 pmol of si-*Alexa* (upper panel) or si-*gfp* (lower panel); fluorescence was recorded in tissues dissected 15-h pi. Images are representative of 20 mosquitoes from two biological replicates.

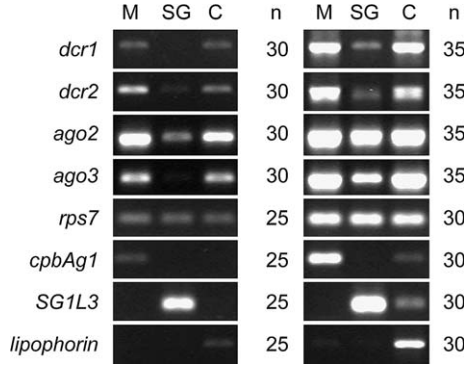

Fig. 2. Expression of the RNAi genes *dicer1*, *dicer2*, *ago2* and *ago3* in *An. gambiae* salivary glands. Total RNA from salivary glands (SG), midguts (M) and carcasses (C: mosquito remnants) of 5-day-old mosquitoes ($n \geqslant 30$) were reverse transcribed and cDNA used for PCR amplification with gene-specific primers (see S2). *rps7* amplification was used as a relative internal control. *cpbAg1*, *SG1L3* and *lipophorin* represent midgut-, salivary gland- and fat body-specific genes, respectively. *n*: cycle numbers.

cells (a cell type involved in immune responses) indicating that these tissues were pervious to siRNA (Fig. 1B). In contrast, no fluorescence could be detected in salivary glands. This suggests that *Anopheles* salivary glands are impervious to siRNA molecules, and presumably to dsRNA, or that they can degrade exogenous dsRNA molecules. Similar results were obtained with *Anopheles stephensi* and *Aedes aegypti* mosquitoes (data not shown).

2.2. Gene silencing in Anopheles salivary glands requires injection of large amounts of dsRNA

Whatever the reason for the absence of RNAi in salivary glands, we tested if the injection of large amounts of dsRNA could result in efficient RNAi. We first targeted *gfp* in the GFP *Anopheles* line by injecting into the haemolymph high amounts of ds-*gfp* (1600 ng/mosquito, i.e., 10 times the amount used previously). Under these conditions, GFP expression in salivary glands was undetectable on day 7 pi as in all other tissues that normally expressed GFP in this line (Fig. 3A). We then targeted the *AgApy* and *SG1L3* genes and quantified gene expression by RT-PCR after injection of various concentrations of dsRNA (Fig. 3, Panels B and C). Expression of both genes could be silenced only when high amounts of the cognate dsRNA were injected into the insect. In addition, the doses of dsRNA necessary for efficient silencing were gene-dependent. For example, whereas injection of 840 ng of ds-*AgApy* induced an 80% reduction in *AgApy* expression, injection of 2300 ng of ds-*SG1L3* was required to produce a similar effect on *SG1L3* expression. As *AgApy* mRNA is less abundant than *SG1L3* mRNA (data not shown), this gene-dependent silencing effect might result from differences in mRNA abundance, but might also reflect differences in the efficiency of the dsRNA sequences to silence their cognate genes. For both genes, the RNAi effect lasted at least 13 days (data not shown).

One possible side effect of injecting such large amounts of dsRNA is loss of specificity. To test this, the protein content of salivary glands isolated from water-, ds-*gfp*- and ds-*AgApy*-injected mosquitoes was compared. No striking difference in protein profiles was detected (Fig. 3D). In addition, *AgApy* mRNA level was not modified in ds-*SG1L3*-injected mosquitoes (Fig. 3E), and similarly *SG1L3* mRNA level was not modified in ds-*AgApy*-injected mosquitoes (Fig. 3F). We therefore conclude that genes expressed in salivary glands can specifically be silenced by injecting large amounts of dsRNA in the mosquito haemocele and that the efficiency of the technique can be gene-dependent.

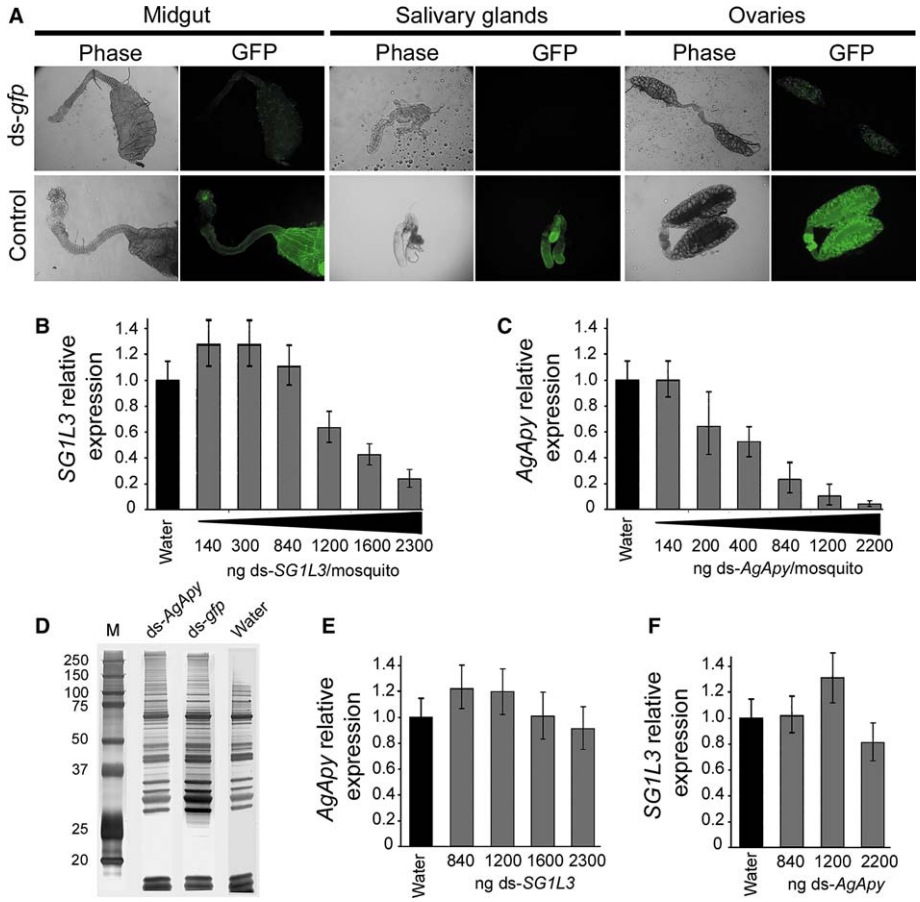

Fig. 3. Efficient gene silencing in *An. gambiae* salivary glands. (A) GFP silencing in the GFP-transgenic *An. gambiae* line. Mosquitoes were injected with 1600 ng ds-*gfp* (upper Panel) or water (lower panel). Fluorescence was monitored 7 days pi. Images are representative of 20 mosquitoes from two biological replicates. (B, C) *SG1L3* and *AgApy* silencing. Mosquitoes were injected with various amounts of ds-*SG1L3* (B) or ds-*AgApy* (C); mRNA was quantified by quantitative RT-PCR on RNA extracted from 10–15 whole mosquitoes 4 days pi. Each experiment corresponds to 3 or 4 biological replicates except for the dose 2300 ng ds-*SG1L3* that was duplicated. (D) SDS–PAGE analysis of salivary gland proteins from ds-*gfp*-, ds-*AgApy*- and water-injected mosquitoes. Salivary glands were isolated 4 days pi (M: molecular weight markers). (E) Quantification of *AgApy* mRNA in ds-*SG1L3*-injected mosquitoes. (F) Quantification of *SG1L3* mRNA in ds-*AgApy*-injected mosquitoes.

2.3. Functional analysis of AgApy

To further test this method for functional analysis of salivary gland genes, we analyzed the effects associated to *AgApy* silencing. *AgApy* was described as a putative apyrase (EC 3.6.1.5) of the 5′ nucleotidase family, based on amino acid sequence similarity to *A. aegypti* apyrase [10,11]. The enzymatic activity that defines apyrase is the hydrolysis of ATP to ADP and ADP to AMP. One well-described biological activity of apyrase is inhibition of platelet aggregation induced by ADP [12]. Apyrase activity is present in the saliva of haematophagous arthropods, and in mosquitoes has been correlated with probing activity, the process that allows mosquitoes to locate blood vessels before blood feeding [13–15].

Mosquitoes were injected with 1200 ng ds-*AgApy*, 1200 ng ds-*gfp* or water and gene expression was quantified 7 days later by RT-PCR. We verified that *AgApy* expression was decreased in ds-*AgApy*-injected mosquitoes (85% reduction) compared to control mosquitoes, and that expression of *AgApyLike1*, which shares 47% identity to *AgApy*, was not modified in ds-*AgApy*-injected mosquitoes (data not shown). Next, salivary gland extracts were assayed for apyrase enzymatic activity and their effect onto platelet aggregation. Apyrase activity in salivary gland extracts from ds-*AgApy*-injected mosquitoes was reduced by 35% compared to the control (Table 1), suggesting that the protein encoded by *AgApy* contributes weakly to apyrase activity. However, despite this moderate contribution of AgApy protein to the apyrase activity, salivary gland extracts from ds-*AgApy*-injected mosquitoes were significantly less efficient at inhibiting platelet aggregation induced by ADP than salivary gland extracts from control mosquitoes (Fig. 4A).

To test if the probing behaviour of mosquitoes is indeed correlated to inhibition of platelet aggregation by mosquito apyrase, we compared the probing behaviour of ds-*AgApy*-injected mosquitoes to control mosquitoes. Indeed, ds-*AgApy*-injected mosquitoes took twice the time of control mosquitoes to probing before blood feeding (Fig. 4B).

Table 1
Mean apyrase activity (mU Pi release/min/gland) in salivary gland of water-, ds-*gfp*- and ds-*AgApy*-injected mosquitoes

Substrate	Control ($n = 4$)	ds-*gfp* ($n = 1$)	ds-*AgApy* ($n = 4$)	Reduction (%) [1-(ds-*AgApy*/control)]
ATP	14.8 (2.2)	15.3	9.7 (1.8)	35
ADP	11.4 (0.7)	11.1	7.0 (1.6)	38
AMP	nd	nd	nd	

One unit corresponds to the release of 1 μmol Pi/min at 37 °C. S.E. is in parentheses. nd: not detectable.

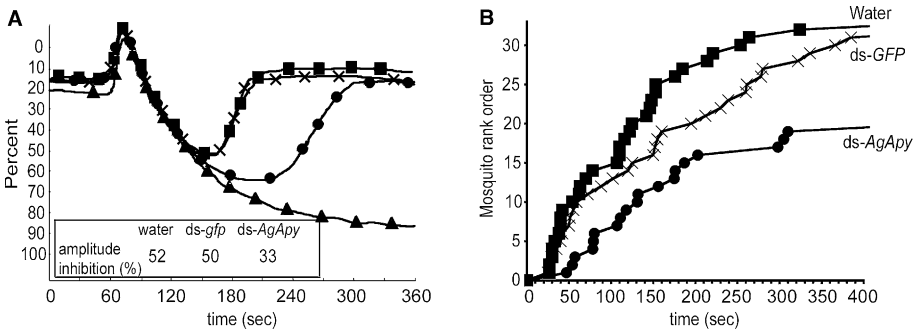

Fig. 4. Functional analysis of *An. gambiae* apyrase by RNAi. (A) In vitro platelet aggregation inhibition by salivary gland extracts. Rabbit platelets were incubated with salivary gland extracts from *An. gambiae* injected with water (■), ds-*gfp* (×) or ds-*AgApy* (●), prior ADP addition (10 μM). ▲: control (no salivary gland extracts). Inhibition was expressed as percentage of maximal aggregation induced by 10 μM ADP. (B) Modification of probing time in ds-*AgApy*-injected mosquitoes. Water-, ds-*gfp*- or ds-*AgApy*-injected mosquitoes were presented 7 days pi to an anesthetized mouse. Mosquitoes starting to feed at any given time were recorded and rank order was plotted against time. Each treatment was assayed in duplicate and curves represent cumulating of at least 39 mosquitoes tested for up to 600 s each. Mosquitoes probing for more than 400 s are not represented on the graph.

These results using a reverse genetic approach demonstrate that *AgApy* encodes an active apyrase and that the level of AgApy in saliva modifies the probing behaviour of mosquitoes. The moderate penetrance of the phenotype associated with ds-*AgApy* mosquitoes might be due to the partial inactivation of *AgApy* expression or redundant activity. As *AgApy* expression was reduced by 85%, our data rather suggest that *An. gambiae* saliva contains other molecules harbouring apyrase activity. These could correspond to the products of *AgApyLike1* and *Ag9* that share similarity to *Aedes* and *Cimex* apyrases, respectively [4,7].

In conclusion, this report demonstrates that injecting dsRNA in the haemolymph of *Anopheles* induces gene silencing in a tissue and dose-dependent manner, and that silencing gene expression in salivary glands requires between 5 and 30 times more dsRNA than in haemocyte or midgut. We provide evidence that injecting such high amounts of dsRNA does not seem to affect the specificity of the silencing process and that this method can be used for functional analysis. Therefore, these results open the way for exploring the abundance of data that are starting to emerge from proteomic and transcriptomic studies of *Anopheles* salivary glands [6,16–20] with a special emphasis on the role of salivary gland components in the maturation and transmission of *Plasmodium* sporozoites.

3. Methods

3.1. Mosquito strains and injection procedures

Anopheles gambiae Yaoundé strain and the transgenic pK(BIGα) line [5] were reared under standard procedures [8]. dsRNA or siRNA solutions were injected into one-day-old cold-anesthetized mosquitoes and a Nanoject micro-injector (Drummond Scientific). Up to 207 nl were injected per mosquito. Control mosquitoes were injected with water.

3.2. Generation of dsRNA and siRNA

cDNA fragments corresponding to the N-terminal sequence of SG1L3 (488 bp) and AgApy (480 bp) were produced by RT-PCR using total RNA extracts from whole mosquitoes and gene-specific primers extended with a T7-promoter sequence containing tail (see S2), and used as template to generate dsRNA by in vitro transcription (Ambion T7 Megascript). E*gfp* DNA fragment was similarly amplified from transgenic pK(BIGα) *An. gambiae* genomic DNA and used for generating dsRNA. dsRNA concentration and quality were estimated by spectrometry at 260 nm (1 OD_{260} = 40 μg RNA/ml) and electrophoresis on an ethidium bromide containing agarose gel. *gfp*-siRNA (si-*gfp*) and Alexa Fluor 555-siRNA (si-*Alexa*), from Qiagen were resuspended at 20 μM in siRNA solution.

3.3. mRNA expression analysis

cDNA were produced as previously described [8]. *SG1L3* and *AgApy* mRNA quantification was performed by quantitative real-time PCR using the $2^{-\Delta\Delta Ct}$ method (User Bulletin 2, ABI). Expression analysis for *dicer1*, *dicer2*, *ago2* and *ago3* was performed by semi-quantitative RT-PCR as detailed in S2.

3.4. Protein analysis

Proteins from isolated salivary glands were extracted with 50 mM Tris, pH 8.0, 125 mM NaCl, 1% glycerol, 1% SDS, 10 mM β-mercaptoethanol, and separated on a 12% SDS–polyacrylamide gel and silver stained.

3.5. Platelet aggregation studies and apyrase enzymatic assay

Salivary glands dissected in 15 mM NaCl were sonicated (10 min, maximum amplitude, Cup horn, Sonics & Materials Inc., USA) and centrifuged (30 min, 130 000 × *g*). Soluble proteins were quantified using the BCA™ protein assay (Pierce, Rockville, IL, USA).

Platelet rich plasma (PRP) and platelet poor plasma (PPP) were prepared from male rabbits (HY/CR strain) as described in [21]. Platelet aggregation was monitored in 450 μl aliquots of PRP by light transmission in an aggregometer (Model 490, Chrono-log corporation, USA) and stirred at 1000 rpm. Extracts corresponding to 1.7 salivary glands were added to PRP and incubated for 1 min at 37 °C before ADP addition (10 μM). In control experiments, salivary gland extracts were substituted by 15 mM NaCl. Optical aggregation results (amplitude) were expressed as percentage of aggregation at a given time interval from reagent addition. Aggregation tests were performed in triplicates for each salivary gland extract and results were expressed as mean of two independent assays.

Apyrase activity was measured as described in www.sigmaaldrich.com/img/assets/18200/Apyrase_ADP_as_Substrate.pdf with assays carried out at 37 °C in buffer containing 5 mM $CaCl_2$, 2 mM of ATP, ADP or AMP, 20 mM glycine, 20 mM Tris, pH 9.0, and serial dilutions of salivary gland extract corresponding to 0.1, 0.5 or 0.01 salivary gland, in 20 μl. Absorbance at $A_{660\,nm}$ was recorded (Spectramax plus; Molecular devices).

3.6. Fluorescence analysis

GFP expression was monitored using a Nikon SMZ1500 binocular. si-*Alexa* fluorescence was analyzed 1–4 h post-dissection at room temperature using an inverted Zeiss microscope with Zeiss Red and Green filters. All images were captured after 0.5 second exposure time with a Nikon camera.

Acknowledgements: We thank R. Ménard and P. Baldacci for critical reading of the manuscript and helpful suggestions. We are grateful to F. Frischknecht, S. Thiberge, B. Martin, R. Amino and C. Lavazec for discussion and comments. We thank members of the CEPIA (Pasteur Institute) for mosquito rearing, and M.Q. Benedict and the MR4 for kindly providing the *An. gambiae* GFP strain. This project was supported by Institut Pasteur fellowship to B.B. and research funds from Pasteur Institute (GPH *Anopheles*) and CNRS (Post-séquençage Anophèles).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.02.069.

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