Insect Biochemistry and Molecular Biology 43 (2013) 146–152

Contents lists available at SciVerse ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Blood meal induced microRNA regulates development and immune associated genes in the Dengue mosquito vector, *Aedes aegypti*

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ARTICLE INFO

Article history:
Received 5 October 2012
Received in revised form
14 November 2012
Accepted 20 November 2012

Keywords:
MicroRNA
Blood meal
Mosquito
aae-miR-375
Gene regulation

ABSTRACT

Aedes aegypti is a blood-feeding mosquito that transmits human pathogens such as Dengue virus, Yellow Fever virus and Chikungunya virus. Recently, dramatic changes in the transcriptome of *Ae. aegypti* following a blood meal have been reported; however, the molecular factors involved in regulating these changes are largely unknown. In this study, we found induction of a number of endogenous microRNAs (miRNAs) in blood fed (BF) mosquitoes. One of these miRNAs, aae-miR-375, was only detected in BF mosquitoes. Based on target analyses, we found six different genes involved in development and immunity being regulated by aae-miR-375 at the post-transcriptional level. We further confirmed the specific interaction of aae-miR-375 with the target sequences in the transcripts of two immune related genes, *cactus* and *REL1*, using a GFP-based reporter assay. Overall, results from this report indicate that miRNAs induced upon blood feeding can regulate the transcript levels of several genes that are important in development and immune responses in mosquitoes. In addition, we demonstrate that aae-miR-375 enhances Dengue virus serotype 2 (DENV-2) infection in an *Ae. aegypti* cell line.

1. Introduction

The mosquito *Aedes aegypti* (yellow fever mosquito) is distributed worldwide and is the principal vector of yellow fever and Dengue virus (DENV) which result in more than 24,000 deaths annually (Clemons et al., 2010). As female *Ae. aegypti* mosquito requires a blood meal for egg development, viruses and parasites present in infected blood are taken up during feeding and infect mosquito midgut epithelium where they replicate, disseminate and eventually infect salivary glands. Mosquitoes are able to transmit pathogens throughout their adult lifespan. Recent studies have shown that blood feeding leads to differential expression of a large number of genes in *Ae. aegypti* and *Anopheles gambiae* salivary glands (Bonizzoni et al., 2011; Das et al., 2010). Blood meal-induced genes were implicated in important biological functions such as digestion, progression of the gonotrophic cycle, insect-specific detoxification, cytoplasmic transport in nurse cells during oogenesis, defense against pathogens, Toll-dependent antimicrobial response, inhibitors of apoptosis and gene silencing.

How induction of these genes occurs after blood feeding is currently unknown but microRNAs (miRNAs), non-coding 18–25 nucleotide RNAs, can potentially regulate more than 60% of genes involved in various processes in almost all metazoans (Friedman et al., 2009). A general model of miRNA biogenesis involves a nuclear step, export and a cytoplasmic step. In the nucleus, RNA polymerase II transcribes the primary miRNA (pri-miRNA; >100 nt) containing one or more hairpin stem loop structures that are then cleaved into 60–110 bp precursor miRNA (pre-miRNA) by Drosha. Pre-miRNA is then exported to the cytoplasm by Exportin-5 where it is processed by Dicer 1 into miRNA/miRNA* duplex (Winter et al., 2009). On the basis of weak 5′ base pairing, one of the strands of this duplex is loaded by Argonaute (Ago) protein and guides RNA Induced Silencing Complex (RISC) to mRNA target sequence (with perfect or partial complementarity) resulting in degradation of mRNA, translational repression or induction in gene expression (Asgari, 2011). Various functional base pair complementarities in mRNA–miRNA interactions have been documented that include seed region (nucleotides 2–8 in the miRNA), centered pairing and complementarity at the 3′ end of miRNA (Bartel, 2004, 2009; Shin et al., 2010).

Previously, we reported differential expression of *Ae. aegypti* miRNAs in the presence of the bacterial endosymbiont *Wolbachia* (Hussain et al., 2011). Here, we investigate differential expression of *Ae. aegypti* miRNAs upon blood feeding with special functional analysis of one miRNA, aae-miR-375, that was exclusively induced

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http://dx.doi.org/10.1016/j.ibmb.2012.11.005

in blood fed mosquitoes. The results suggest that aae-miR-375 may regulate several genes associated with development and immunity.

2. Materials and methods

2.1. Insect cells, mosquitoes and DENV infection

Ae. aegypti Aag2 cells were maintained in growth media in a 1:1 mixture of Mitsuhashi–Maramorosch and Schneider's insect media (Invitrogen), supplemented with 10% FBS. *Ae. aegypti* were reared at 25 °C with 80% relative humidity and a 12-h light regime. Larvae were maintained with fish food pellets (TetraMin; Tetra) and adults were offered 10% sucrose solution and human blood. Mosquitoes infected with the *w*MelPop-CLA strain of *Wolbachia* (+Wol) were used as previously described (Hussain et al., 2011). For DENV infection, Aag2 cells were seeded in 12-well plates to a confluency of about 80%. DENV2 New Guinea C (NGC) of 0.01 multiplicity of infection (MOI) diluted in the growth medium (mentioned above) was then added to the cells. After rocking at room temperature for 1 h, the plates were incubated at 28 °C for 72 h.

2.2. Northern blot hybridizations

Small RNA was isolated from total RNA extracted from mosquitoes using the PureLink miRNA isolation kit (Invitrogen). Small RNA samples (20 μg) were run on 15% urea denaturing polyacrylamide gels, electroblotted to nylon membranes by a semi-dried Western blotting apparatus (Bio-Rad), and UV cross-linked. DNA oligonucleotides (20–23 mer) reverse complementary to specific miRNA sequences were labelled with [α-^{32}P]-dCTP using terminal nucleotide transferase. All probe hybridizations (in 1× SSC + 1% SDS) and washes (3× SSC + 5% SDS twice and 1× SSC + 1% SDS twice) were done under stringent conditions at 50 °C. Blots were exposed to a phosphorimager screen overnight, and radioactive signals were detected using a phosphorimager scanner (Storm). Blots were washed for removing old probe with boiling 0.1% SDS twice for 30 min each time. Stripping of the probe was confirmed by scanning the blots as described above.

2.3. miRNA target studies and RT-qPCR

NCBI BLAST and subsequently RNAHybrid software were used to find potential targets of aae-miR-375 in the *Ae. aegypti* genome. Complementarity of the seed region and minimum free energy (mfe) of less than −23 kcal/mol were the main criteria used for selection of targets. The transcript levels of target genes and *GFP* were analysed by RT-qPCR using gene-specific primers while utilising the mosquito gene *RPS17* as reference. For each experiment, two or three biological replicates with three technical replicates were analysed in a Rotor-Gene thermal cycler (QIAGEN) under the following conditions: 95 °C for 30 s, and 40 cycles of 95 °C for 10 s and 60 °C for 45 s, followed by the melting curve (68 °C–95 °C). *t*-test or ANOVA was used to compare differences in means between different treatments. Target gene sequences of *cactus* and *REL1* were PCR amplified with specific primers and cloned into pIZ/V5-His vector (Invitrogen) upstream to *GFP* using *Hind*III and *Bam*H1 restriction sites, resulting in pIZ/cac-GFP and pIZ/rel-GFP. aae-miR-375 mimic (UUUGUUCGUUUGGCUCGAGUUA), aae-miR-375 mutant mimic (UUUGCCCGUUUGGCUCGAGUUA; mutated residues are underlined) and control scrambled mimic (UUCUCCGAACGUGUCACGUTT) were synthesized by Genepharma and used in transfections in Aag2 cell line using the Cellfectin reagent (Invitrogen). Mimics are chemically synthesized double-stranded RNAs which mimic mature endogenous miRNAs after transfection into cells.

2.4. Mosquito injection

Injection of aae-miR-375 mimic, control mimic and water control was carried out in the thorax of 4 day-old CO_2-anesthetized female mosquitoes (Wol−). For the mimic and control, 2 μl of a 100 μM/ml solution in sterile water was injected into each mosquito. 24 h after injection, surviving female mosquitoes were given access to a human blood feed (Monash University Human Ethics Approval # CF11/0766 2011000387). A further 24 h later, blood fed (BF) mosquitoes were separated from non-blood fed (NBF) and both were provided with a 10% sucrose solution. Mosquitoes were collected at 3 (8-day-old) and 7 (12-day-old) days post-blood feed and stored at −80 °C until RNA extraction.

3. Results

3.1. Ae. aegypti miRNAs are up-regulated upon blood feeding

Considering that blood feeding leads to massive up-regulation of several transcripts in *Ae. aegypti* (Bonizzoni et al., 2011), we compared differential expression of miRNAs in blood fed (BF) and non-blood fed (NBF) mosquitoes at days 8 and 12 post-pupal emergence (3 and 7 days post-blood feeding in BF, respectively). To determine the relationship with *Wolbachia* infection, we compared *w*MelPop-CLA infected mosquitoes (+Wol) and tetracycline-treated mosquitoes (−Wol). We selected seven highly expressed miRNAs that have been shown to be important in various biological functions in insects and other animals (Chawla and Sokol, 2011). These were aae-miR-375, -2940*, -125, -317, -14, miR-1 and bantam. In Northern blot hybridizations, all seven miRNAs showed significantly greater induction in BF mosquitoes when compared to NBF. However, aae-miR-2940* was found to be induced at the same level in BF and +Wol mosquitoes at both 8d and 12d, and miR-125 was also induced in 8d +Wol mosquitoes more than BF mosquitoes (Fig. 1A). Most of the miRNAs were also found in higher quantities in +Wol mosquitoes compared to −Wol mosquitoes (Fig. 1A). Interestingly, only aae-miR-375 was exclusively detected in BF mosquitoes and expression was further increased in 12d BF mosquitoes suggesting both stability as well as functionality of this miRNA. In further Northern blot analyses, we also compared sugar fed (SF) mosquitoes at 8d and 12d following emergence with BF mosquitoes of the same age and detected miR-375 only in BF mosquitoes, confirming this miRNA is only induced after blood feeding (Fig. 1B). Moreover, we could not detect miR-375 in Aag2 (from *Ae. aegypti*) and C6/36 (*Aedes albopictus*) cells (data not shown), which gives further evidence that this miRNA is involved in blood feeding.

3.2. aae-miR-375 regulates several genes

Exclusive induction of aae-miR-375 in BF mosquitoes led us to investigate its potential target genes in *Ae. aegypti*. Six different candidate target genes were identified in NCBI BLAST searches of the *Ae. aegypti* genome against the aae-miR-375 sequence which were further confirmed using the RNAHybrid software (Kruger and Rehmsmeier, 2006) (Table 1). We found good target sequences with minimum free energy (mfe) of more than −23 kcal/mol in the 5′UTR of *cactus*, *REL1* and *prohibitin*, in the 3′UTR of *DEAD box ATP-dependent RNA helicase* and a hypothetical protein, and the coding region of *kinesin*. In these targets, there was complete seed region complementarity except in *prohibitin* with one nucleotide mismatch and in *Kinesin* where there is strong central pairing. In order to analyse transcriptional changes of the target genes, we injected a synthetic aae-miR-375 mimic and a control mimic of random sequences in NBF female mosquitoes and extracted RNA after 3 days