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Regulation of atrophin by both strands of the mir-8 precursor

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ABSTRACT

In *Drosophila melanogaster*, miR-8-3p regulates mRNA levels of atrophin, a factor involved in neuromotor coordination, and we found that *Blattella germanica* with suppressed atrophin showed motor problems. Bionformatic predictions and luciferase-reporter tests indicated that *B. germanica* atrophin mRNA contains target sites for miR-8-3p and miR-8-5p. Suppression of miR-8-3p or miR-8-5p appeared to increase atrophin mRNA. The effects of suppression of Argonaute (AGO) 1 or AGO2 expression on miR-8-3p and miR-8-5p suggested that miR-8-3-p might predominantly bind to AGO1, whereas miR-8-5p might bind to a moderate extent to both AGO1 and AGO2 in the respective RNA-induced silencing complexes (RISCs). We propose that the interplay of miR-8-3p, miR-8-5p, AGO1 and AGO2, maintain the appropriate levels of atrophin mRNA. This would be the first example of two strands of the same miRNA precursor regulating a single transcript.

1. Introduction

One of the most relevant recent advances in the field of regulation of gene expression is the discovery of microRNAs (miRNAs) in the nematode *Caenorhabditis elegans* (Lee et al., 1993). Subsequently, the occurrence of miRNAs has been reported in almost all animal and plant groups (Griffiths-Jones et al., 2008) and their role as key protagonists of an additional layer of regulation of gene expression is now widely recognized (Bartel, 2004). Today, miRNAs can be defined as endogenous short noncoding RNAs of 21–23 nucleotides that generally regulate a target mRNA by blocking its translation and even by degrading it. With this mechanism, miRNAs sculpt mRNA and protein patterns, confer robustness to biological processes by refining transcriptional programs and attenuating aberrant transcripts, and may help to suppress random fluctuations in transcript copy number. For these reasons, miRNAs have been qualified as "fine-tuning" molecules (Bartel, 2004), and their importance has been recognized not only in normal development and physiology, but also in medical sciences and in evolution (Christodoulou et al., 2010; Ebert and Sharp, 2012).

Mature miRNAs are processed from the corresponding hairpin miRNA precursor by the RNase III enzyme Dicer yielding two partially complementary single strand miRNAs: the "mature" miRNA and the "passenger" strand (Kim et al., 2009). In insects, the so-called mature miRNA is generally incorporated into the RNA-induced silencing complex (RISC) combined with the protein Argonaute (AGO) 1, which has endonuclease activity directed against mRNA strands that display extensive complementarity to their bound miRNA. The miRNA then guides the AGO1-RISC to the target mRNA, binds to the imperfectly-paired miRNA sites located within it, and blocks transcript translation, eventually leading to mRNA degradation, according to the typical mode of action of miRNAs (Bartel, 2004; Belles et al., 2012). The so-called passenger strand is generally degraded, but in some cases it can be incorporated into a AGO2-RISC, which is optimized for target mRNA degradation (Czech et al., 2009; Okamura et al., 2009).

In an 11-million reads miRNA library from whole-body sixth instar female nymphs of the cockroach *Blattella germanica*, we noticed a number of cases where the "passenger" strand of a given miRNA was as abundant, or even more abundant, than the corresponding "mature" miRNA (Cristino et al., 2011), which suggested that the two strands of the same precursor give functional miRNAs. This was not surprising, given that previous investigations in vertebrates (Yang et al., 2011) and in the fly *Drosophila melanogaster* (Okamura et al., 2008) had shown that a significant number of "passenger" miRNAs are structurally conserved in diverse animal groups, suggesting that they function as miRNAs. In our miRNA library, the case of miR-8 was of particular interest because the

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"mature" miR-8 (actually, miR-8-3p) was represented by 4906 reads, whereas for the "passenger" strand (miR-8-5p), 47,160 reads were counted (Cristino et al., 2011). In *D. melanogaster*, miR-8-5p is also highly expressed, but the relative expression levels are still 40-fold higher in the case of miR-8-3p (Berezikov et al., 2011). The question that intrigued us was whether miR-8-5p might function as a miRNA in *B. germanica*.

A search for miR-8 target candidates in insects revealed a paper by Stephen Cohen and his coworkers (Karres et al., 2007) who elegantly demonstrated that miR-8 (indeed, miR-8-3p) regulates mRNA levels of atrophin in *D. melanogaster*. *Drosophila* atrophin is related to the atrophin family of mammalian transcriptional co-repressors that play key roles in developmental and neural processes (Wang and Tsai, 2008). Cohen's team identified *Drosophila* atrophin as a direct target of miR-8-3p, and showed that mutant miR-8-3p phenotypes had high atrophin activity, which resulted in elevated brain apoptosis and in neuromotor coordination defects (Karres et al., 2007). With these antecedents, we proceeded with our cockroach model by cloning and characterizing, in structural and functional terms, the cDNA or RNA corresponding to the main players that might be involved in the above process in question: atrophin, the miR-8 precursor and its two strands miR-8-3p and miR-8-5p, and the associated AGO1 and AGO2 proteins.

2. Materials and methods

2.1. Insects

Freshly ecdysed sixth (last) instar nymphs of the cockroach *B. germanica* were obtained from a colony reared in the dark at 30 ± 1 °C and 60–70% relative humidity. The entire animal except the head (to avoid interferences with the eye pigments) and the digestive tube (to avoid contamination with parasites) was used for RNA extractions. All dissections were carried out on carbon dioxide-anaesthetized individuals. Samples for RNA extraction and quantification were frozen immediately after dissection, and stored at −80 °C until use. RNA isolation was carried out with miRNeasy® Mini Kit (QIAGEN), which increases the yield of small RNAs. Reverse transcription was carried out with the NCode™ miRNA First-Strand cDNA Synthesis and qRT-PCR Kits (Invitrogen), which allows the quantification of large and small RNAs by real-time PCR.

2.2. Cloning the miR-8 precursor

Using the sequences of miR-8-5p and miR-8-3p as primers and cDNA from last instar female nymphs of *B. germanica* as template, we amplified a fragment of 59 bp, which was subcloned into the pSTBlue-1 vector (Novagen) and sequenced. The RNA fold algorithm of Gruber et al. (2008) was used to predict if the primary sequences obtained folded following the typical hairpin structure of a pre-miRNA.

2.3. Cloning atrophin cDNA

A partial sequence of *B. germanica* atrophin, including the 3′UTR, was obtained following a RT-PCR strategy using degenerate primers designed on the basis of conserved motifs from insect atrophin sequences and cDNA from last instar female nymphs of *B. germanica* as a template. The sequence of the amplified fragment (973 bp) was identified as the equivalent region in known insect atrophin sequences. Then, the 3′ fragment of the sequence was completed by 3′ RACE (3′-RACE System Version 2.0; Invitrogen) using the same template, thus obtaining a final fragment of 1820 bp (GenBank accession number HF912426). PCR products were subcloned and sequenced as described above. Primer sequences are indicated in the Table S1.

2.4. Cloning AGO1 and AGO2 cDNAs

The *B. germanica* AGO1 and AGO2 were obtained following a RT-PCR strategy using specific primers based on sequences obtained from *B. germanica* transcriptomes of whole body of sixth instar female nymph, and cDNA from the same instar and sex as template. We further extended the sequence by subsequent 3′ and 5′ RACEs using cDNA from last instar female nymphs, and we obtained a sequence of 2834 bp length for AGO1 and of 3361 bp length for AGO2 (GenBank accession numbers HF912424 and HF912425, respectively). PCR products were subcloned into the pSTBlue-1 vector (Novagen) and sequenced. Primer sequences are indicated in the Table S1.

2.5. Quantification of miRNAs and mRNAs by quantitative real-time PCR

For miRNA and mRNA expression studies by quantitative real-time PCR (qRT-PCR), 400 ng of total RNA were reverse transcribed using the NCodeTM First-Strand cDNA Synthesis Kit (Invitrogen), which works for both miRNA and mRNA quantification, following the manufacturer's protocols. Amplification reactions were carried out using IQTM SYBR Green Supermix (BioRad) and the following protocol: 95 °C for 2 min, and 40 cycles of 95 °C for 15 s and 60 °C for 30 s, in a MyIQ Real-Time PCR Detection System (BioRad). After the amplification phase, a dissociation curve was obtained in order to ensure that there was only one product amplified. All reactions were run in triplicate. Statistical analysis of relative expression results was carried out with the REST software tool (Pfaffl et al., 2002). Results are given as copies of RNA per 1000 copies of U6. Primer sequences for the mRNAs are indicated in the Table S2.

2.6. Prediction of miRNA binding sites

To predict miR-8-3p and miR-8-5p binding sites in the 3′-UTR of atrophin with the highest and comparable reliability, we used the following algorithms and parameter sets. RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) (Rehmsmeier et al., 2004), with a distribution probability of parameter $\xi = 1.98$ and $\Theta = 1.16$; miRanda (http://www.microrna.org) (Enright et al., 2003), with a score threshold of 100; PITA (http://genie.weizmann.ac.il/index.html) (Kertesz et al., 2007), with the seed limitation between 5 and 8; and miRiam (http://ferrolab.dmi.unict.it/miriam.html) (Lagana et al., 2010), with the highest sensibility parameters of the setting.

2.7. Luciferase assay to assess the occurrence of miR-8-3p and miR-8-5p sites

Luciferase reporters were generated by PCR amplification of a DNA fragment corresponding to the 3′UTR of the atrophin transcript from pSTBlue-1 vector (Novagen) using the primers: Atroph-Fwd and Atroph-Rev (primer sequences indicated in the Table S3), and cloned under the control of the tubulin promoter downstream of the Firefly luciferase coding region between the SpeI and XhoI sites of plasmid pJ-Luc described elsewhere (Rehwinkel et al., 2005). All PCR fragments were cloned SpeI-XhoI in the pJ-Luc vector and confirmed by sequencing. To study the expression of miR-8, a fragment of 272 bp encompassing the miR-8 hairpin was amplified by PCR with the primers: miR-8-Fwd and miR-8-Rev (primer sequences indicated in the Table S3) from *D. melanogaster* genomic DNA and cloned in vector pAc5.1 (Actin Promoter)