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SHORT COMMUNICATION

Super-induction of Dicer-2 expression by alien double-stranded RNAs: an evolutionary ancient response to viral infection?

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Abstract Dicer-2 is a ribonuclease involved in the insect RNAi pathway. On attempting to knockdown Dicer-2 expression in the insect *Blattella germanica* by RNAi, we found that treatment with Dicer-2 dsRNA upregulated the targeted mRNA. This unexpected result was also observed after treating with a nucleopolyhedrovirus dsRNA. Experiments with this alien dsRNA showed an all-or-none response with a threshold for inducing Dicer-2 upregulation between 0.4 and 0.04 μg in terms of dsRNA concentration and between 50 and 20 bp in terms of dsRNA length. The response seems specific of dsRNA given that equivalent experiments carried out with dsDNA did not affect Dicer-2 expression. In insects, Dicer-2 is postulated to be a sensor of viral infections and a key antiviral defense element. The upregulation of Dicer-2 expression after dsRNA administration fits well with this sensor role, and the occurrence of this mechanism in *B. germanica*, a phylogenetically basal insect, suggests that sensing alien RNAs might be an ancestral function of Dicer-2 proteins.

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Introduction

One of the most profound recent discoveries of the biological sciences has been RNA interference (RNAi), by which introduced double-stranded RNA (dsRNA) is diced into a pool of 21-nucleotide small interfering RNA (siRNAs) duplexes that select the target mRNA by base-pairing and destroy it (Meister and Tuschl 2004). Among other utilities, RNAi has become a powerful tool to unveil gene functions in nonmodel insects, thus breaking the *Drosophila* paradigm of functional genomics (Belles 2010). Of nonmodel insects, the cockroach *Blattella germanica* stands among the species most sensitive to RNAi. In addition, it is a hemimetabolan Polyneopteran insect that shows few derived characters, thus representing a "primitive" insect.

In the context of the high-RNAi sensitivity *B. germanica*, we investigated the Dicer ribonuclease involved in the processing of dsRNAs into siRNAs. *Drosophila melanogaster*, a holometabolan Panorpidan species that shows many highly derived characters, has two Dicer ribonucleases. Dicer-1 is involved in the miRNA pathway, processing miRNA precursors into mature miRNAs; while Dicer-2 is involved in the RNAi pathway, processing dsRNAs into siRNAs (Lee et al. 2004). The occurrence of two Dicer ribonucleases in the same species has been reported in other holometabolan insects, which contrasts with the nematode *Caenorhabditis elegans*, another well-studied model from a functional genomics point of view, which has a single Dicer protein that is involved in both RNAi and miRNA pathways (Ketting et al. 2001).

Previously, we characterized a Dicer-1 homolog in *B. germanica* and found, as expected, that it is involved in transforming miRNA precursors into mature miRNAs (Gomez-Orte and Belles 2009). For the present work, we first determined that *B. germanica* also has a Dicer-2 homolog that is distinct from Dicer-1. We then followed a strategy of silencing Dicer-2 expression by RNAi to demonstrate its involvement in the RNAi pathway. We expected that RNAi experiments targeting other mRNAs would not work in these specimens. The results obtained, however, turned out to be quite a contrary to those expected, as treatment with dsRNA targeting Dicer-2 elicited a fast and dramatic upregulation of the targeted mRNA. The same was observed when using alien dsRNAs based on nucleopolyhedrovirus sequences, which suggested to us that the observed upregulation of Dicer-2 expression is a first response to RNA infection.

Material and methods

Insects

The specimens of *B. germanica* used in the experiments were obtained from a colony reared in the dark at 30±1°C and 60–70 % RH. They were anesthetized with carbon dioxide prior to injection treatments and tissue sampling. If not stated otherwise, RNA extractions and transcript measurements were based on the whole body.

Cloning of BgDcr2 cDNA

The *B. germanica* Dicer-2 homolog was obtained following a RT-PCR strategy using degenerate primers designed on the basis of conserved motifs from insect Dicer-2 sequences. As template, we used cDNA of 5–6-day-old adult ovaries obtained from a female that had been RNAi-treated targeting Dicer-1, as previously described (Gomez-Orte and Belles 2009). The sequence of the amplified fragment (1,320 bp) was highly similar to the equivalent region in known insect Dicer-2 sequences. Then, the sequence was completed by 5′ and 3′ rapid amplification of cDNA ends (RACE) (5′- and 3′-RACE System Version 2.0; Invitrogen) using the same template. All PCR products were subcloned into the pSTBlue-1 vector (Novagen) and sequenced. Degenerate primers used in the first amplification and specific primers used in the 3′- and 5′-RACE experiments are available upon request.

RNA extraction and retrotranscription to cDNA

All RNA extractions were performed using the GenElute Mammalian Total RNA kit (Sigma). A 500-ng sample from each RNA extraction was treated with DNAse (Promega) and was reverse-transcribed using the NCode Kit (Roche). RNA quantity and quality was estimated by spectrophotometric absorption at 260 nm using a Nanodrop Spectrophotometer ND-1000® (NanoDrop Technologies).

Determination of mRNA levels by quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) reactions were carried out in triplicate in an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories) using SYBR®Green (Power SYBR® Green PCR Master Mix; Applied Biosystems). A template-free control was included in all batches. The efficiency of the primer sets to measure Dicer-1 and Dicer-2 mRNA levels (primer sequences are available upon request) was first validated by constructing a standard curve through four serial dilutions. mRNA levels were calculated relative to BgActin-5c (accession number AJ862721) expression, using the Bio-Rad iQ5 Standard Edition Optical System Software (version 2.0). Results are given as copies of mRNA per 1,000 copies of BgActin-5c mRNA.

RNA interference

The detailed procedures for RNAi experiments were as described previously (Gomez-Orte and Belles 2009; Lozano and Belles 2011). A dsRNA encompassing a 391-bp fragment located between nucleotides 920 and 1,310 of the Dicer-2, ORF sequence (accession number HE647851) (dsDcr2 in Fig. 1a) was designed to carry out the RNAi experiments. The 343-bp dsRNA targeting Dicer-1 (dsDcr1 in Fig. 1a) was that described by Gomez-Orte and Belles (2009) as "dsBgDcr1-A". The primers used to generate the fragments to prepare dsDcr2 and dsDcr1 are available upon request. The fragments were amplified by PCR and cloned into the pSTBlue-1 vector. For the experiments using a dsRNA sequence from *Autographa californica* nucleopolyhedrovirus (accession number K01149), the following dsRNA lengths were used: 300 bp (from nucleotide 236 to 535), 150 bp (from nucleotide 236 to 285), 50 bp (from nucleotide 236 to 285), and 20 bp (from nucleotide 236 to 255). The dsRNAs were prepared as reported previously (Gomez-Orte and Belles 2009; Lozano and Belles 2011), except that of 20 bp, which was prepared by directly annealing the commercial oligonucleotides CCUACGUGUACGACAACAAG and CUUGUUGUCGUACACGUAGG, which encompass the chosen region. dsDNA of *A. californica* nucleopolyhedrovirus was prepared by PCR amplification of the 300-bp fragment described above (from nucleotide 236 to 535) over PolyH plasmid extraction. A volume of 1 μL of dsRNA (or dsDNA) solution (4 μg/μL, if not stated otherwise) was injected into the abdomen of freshly emerged fifth instar

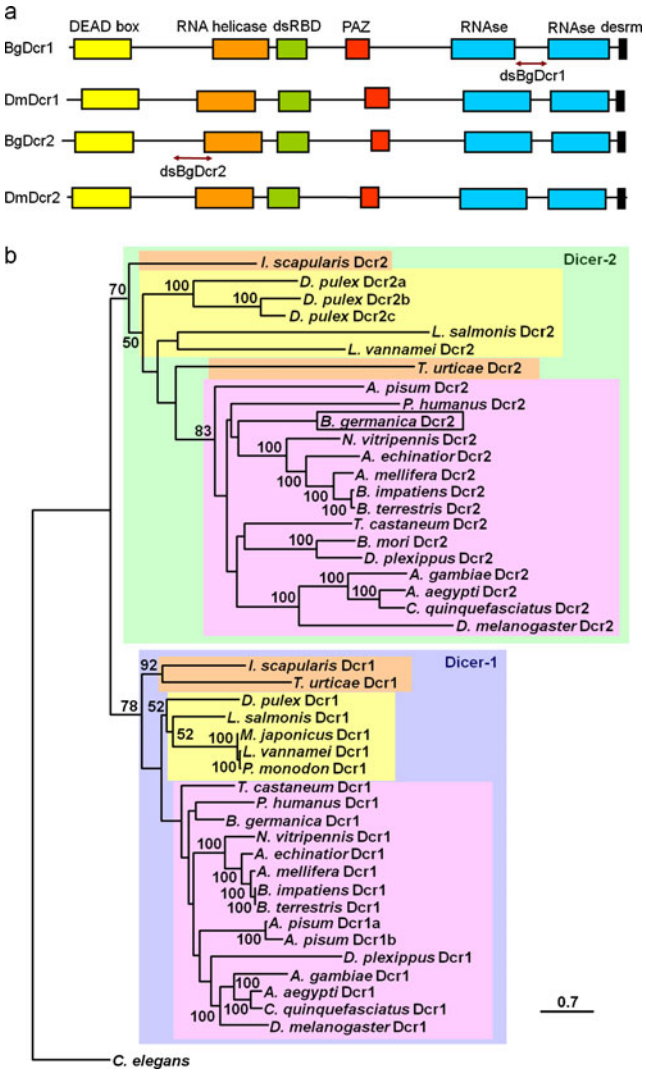

Fig. 1 Organization of *B. germanica* Dicer-2 and phylogenetic relationships with other insect orthologues.**a** Protein organization of *B. germanica* Dicer-2 (BgDcr2) compared with that of Dicer-1 (BgDcr1) reported by Gomez-Orte and Belles (2009), indicating the typical Dicer domains: an N-terminal DEAD box, an RNA helicase, a divergent dsRNAs-binding domain, a PAZ domain, two ribonuclease (RNase III) domains, and an additional dsRNAs-binding domain (desrm), as well as the region encompassed by the dsRNAs (dsDcr1 and dsDcr2) used. Organization of *D. melanogaster* Dicer-1 (DmDcr1) and Dicer-2 (DmDcr2) is also represented for comparison; the DEAD box in DmDcr1 is not recognized by ScanProsite, but conserved residues are identified in the alignments. **b** Maximum likelihood tree of the Dicer protein sequences of arthropod species and the nematode *C. elegans* used as out-group. Complete binomial nomenclature of all species is indicated in "Materials and methods". *B. germanica* Dcr2 sequence described herein is squared; *blue* and *green backgrounds* indicate Dicer- 2 and Dicer-1 sequences, respectively; arachnid, crustacean, and insect species are indicated with *orange*, *yellow*, and *magenta backgrounds*, respectively; bootstrap values >50 are indicated on the corresponding node; the *scale bar* represents 0.7 substitutions per position

female nymphs. Control specimens were treated with the same volume of water.

Sequence comparisons and phylogenetic analysis

We obtained the arthropod sequences labeled as Dicer from GenBank, and the list was enlarged by BLAST search using the *B. germanica* Dicer-1 and Dicer-2 sequences as queries. Two Dicer sequences (tetur19g00520 and tetur07g00990) were obtained from the *Tetranychus urticae* sequenced genome (http://bioinformatics.psb.ugent.be/webtools/bogas). Finally, the species and protein sequences of Dicer-1 (Dcr1) and Dicer-2 (Dcr2) included in the analysis were the following (the accession number or the bibliographic reference indicated in parenthesis; the annotation of Dcr1 or Dcr2 reflects the result of the phylogenetic analysis performed in the present work, not necessarily the annotation stated in the GenBank file). Insects: *Aedes aegypti* Dcr1 (XP_001659747.1) and Dcr2 (AAW48725.1), *Acromyrmex echinatior* Dcr1 (EGI60563.1) and Dcr2 (EGI69620.1), *Anopheles gambiae* Dcr1 (AAO73809.1) and Dcr2 (XP_320248.4), *Apis mellifera* Dcr1 (NP_001116485.1) and Dcr2 (XR_120636.1), *Acyrthosiphon pisum* Dcr1a and Dcr1b (Jaubert-Possamai et al. 2010) and Dcr2 (XP_001945890.2), *B. germanica* Dcr1 (CAX68236.1) and Dcr2 (CCF23094.1), *Bombus impatiens* Dcr1 (XP_003493408.1) and Dcr2 (XP_003485689.1), *Bombus terrestris* Dcr1 (XP_003401955.1) and Dcr2 (XP_003394821.1), *Bombyx mori* Dcr2 (NP_001180543.1), *Culex quinquefasciatus* Dcr1 (XP_001844757.1) and Dcr2 (XP_001855187.1), *D. melanogaster* Dcr1 (NP_524453.1) and Dcr2 (NP_523778.2), *Danaus plexippus* Dcr1 (EHJ64690.1) and Dcr2 (EHJ65725.1), *Nasonia vitripennis* Dcr1 (XP_001605287.1) and Dcr2 (XP_001602524.2), *Pediculus humanus* Dcr1 (XP_002429494.1) and Dcr2 (XP_002430037.1), and *Tribolium castaneum* Dcr1 (EFA11550.1) and Dcr2 (NP_001107840.1). Crustaceans: *Daphnia pulex* (EFX72380.1) Dcr1, Dcr2a (EFX69538.1), Dcr2b (EFX86072.1), and Dcr2c (EFX87988.1); *Lepeophtheirus salmonis* Dcr1 (JP311757.1) and Dcr2 (assembly JP310136.1+JP312169.1); *Litopenaeus vannamei* Dcr1 (ACF96960.1) and Dcr2 (AEB54796.1), *Marsupenaeus japonicus* Dcr1 (ADB44075.1), and *Penaeus monodon* Dcr1 (ABR14013.1). Arachnids: *Ixodes scapularis* Dcr1 (XP_002408100) and Dcr2 (XP_002408099), and *T. urticae* Dcr1 (tetur19g00520) and Dcr2 (tetur07g00990). The unique Dicer sequence from the nematode *C. elegans* (NP_498761.1) was used as out-group.

The protein sequences were aligned using the MAFFT program (http://mafft.cbrc.jp/alignment/software), with the E-INS-I parameter, which is recommended for multidomain proteins. The model of protein evolution that best fits the data was determined using ProtTest 2.4 (http://darwin.uvigo.es/software/prottest.html). The LG+I+G+F evolutionary model was preferred by the ProtTest program and was implemented in

the maximum likelihood analyses. These were carried out by using the PHYML version 3.0 program (http://www.atgc-montpellier.fr/phyml/) with the above evolutionary model. Data were bootstrapped for 100 replicates using the same program.

Results and discussion

Blattella germanica has two Dicer ribonucleases

Cloning of Dicer-2 cDNA in *B. germanica* was accomplished by obtaining a partial sequence by RT-PCR using degenerate primers based on Dicer-2 conserved motifs, from which extended sequence was generated by 5′-RACE and 3′-RACE. These amplifications rendered a cDNA of 5,429 bp (GenBank accession number HE647851). The putative start and stop codons are preceded and followed, respectively, by in-frame stop codons, suggesting that a full-length open reading frame had been obtained. Database BLAST searches suggested that it encoded an orthologue of Dicer-2. The conceptual translation rendered a 1,649-amino acid sequence, and a ScanProsite search revealed that the protein is organized as a typical Dicer sequence (de Jong et al. 2009) with an N-terminal DEAD box, an RNA helicase domain, a divergent dsRNAs-binding domain, a Piwi-Argonaute-Zwille (PAZ) domain, two ribonuclease (RNase III) domains, and an additional dsRNAs-binding domain (desrm) (Fig. 1a). The *B. germanica* Dicer-1 protein previously described (Gomez-Orte and Belles 2009) has the same organization (Fig. 1a) but very different sequence, showing only 27.1 % identity compared with the Dicer-2 reported herein.

The landmark paper of Lee et al. (2004) demonstrated that the two Dicer proteins of *D. melanogaster* were involved in different functions: Dicer-1 in miRNA biogenesis and Dicer-2 in dsRNA processing. Later, Dicer-1 and Dicer-2 proteins have been found to be present in other insects, and our present work reveals, thus, that *B. germanica* also has a Dicer-2 homolog.

Arthropods have Dicer-1 and Dicer-2 ribonucleases

Maximum likelihood phylogenetic analysis of arthropod Dicer protein sequences, using the Dicer orthologue from the nematode *C. elegans* as out-group, rendered a tree (Fig. 1b) that separates Dicer-1 and Dicer-2 sequences into two different groups. The tree clearly shows that the sequence of *B. germanica* reported herein clusters in the Dicer-2 group, while the Dicer-1 previously reported (Gomez-Orte and Belles 2009) clusters in the other group. The data show that arachnids like the blacklegged tick *I. scapularis* and the red spider mite *T. urticae* (Acari) possess Dicer-1 and Dicer-2 genes. The same occurs in crustaceans like the whiteleg shrimp *L. vannamei* (Malacostraca, Decapoda), the sea louse *L. salmonis* (Maxillopoda, Copepoda), and the water flea *D. pulex* (Branchiopoda, Cladocera). Interestingly, *D. pulex* has three paralogues of Dicer-2, which should derive from two Dicer-2 duplications in the Branchiopoda lineage. We presume that Dicer-2 must also occur in those crustaceans where only Dicer-1 has been searched and reported (the decapodans *P. monodon* and *M. japonicus*). In insects, both Dicer-1 and Dicer-2 are generally present in the same species. Of note, and as reported by Jaubert-Possamai et al. (2010), the pea aphid *A. pisum* has two paralogues of Dicer-1. The functional significance of Dicer-1 and Dicer-2 paralogues remains unknown. With few exceptions, the topology of the respective Dicer-1 and Dicer-2 groups approximately follows the currently established phylogenetic relationships of the represented arthropod classes and orders, especially in Dicer-1 group, where, in addition, the sequences have diverged less.

Dicer proteins constitute a widely conserved family that occur in many organisms including plants, fungi, and metazoans. A recent study by de Jong et al. (2009) showed that "basal" (early branching) metazoans like Placozoans and Poriferans have five Dicer proteins, Cnidarians have two, whereas "higher" metazoans have only one, with the exception of insects, which possess two (de Jong et al. 2009). In order to explain the present Dicer diversity within different groups, these authors postulated an ancient duplication event of a "Proto-Dicer" gene at the origin of metazoans followed by successive lineage-specific duplications. De Jong et al. (2009) did not include noninsect arthropods in their analysis, and they proposed that the occurrence of the Dicer-1 and Dicer-2 genes in insects should be explained by a duplication that occurred in the lineage leading to this metazoan class (de Jong et al. 2009). In our phylogenetic study, we have considered all data available in arthropods and found that there are Dicer-1 and Dicer-2 sequences not only in insects but also in crustaceans (sampled in Malacostraca, Maxillopoda, and Branchiopoda) and in arachnids (sampled in Acari). No Dicer sequences have yet been described from miriapods. However, if the Mandibulata hypothesis is followed (Giribet and Edgecombe 2012), by which the Myriapoda clusters with the Crustacea and Hexapoda while the Chelicerata is a sister group of them all, then the most parsimonious prediction is that miriapods would have Dicer-1 and Dicer-2 genes, and that the gene duplication traces to the origin of the arthropods during the Cambrian.

Injection of dsRNA to target Dicer mRNA induces Dicer-2 upregulation

As an initial Dicer-2 RNAi experiment, we performed a "conventional" treatment of 4 μg of a 391-bp dsRNA targeting Dicer-2 mRNA (dsDcr2) injected into freshly emerged fifth instar female nymphs. Specimens of the same sex and age treated with the same volume (1 μL) of water were used

as controls. We then quantified Dicer-2 mRNA at defined intervals during the entire instar in treated and control samples. Unexpectedly, we observed a dramatic (fivefold) upregulation of Dicer-2 (the targeted mRNA) 6 h after the treatment (Fig. 2a). One day after the treatment, Dicer-2 mRNA levels were similar to controls, and when subsequently measured every 24 h, the levels were also approximately similar to controls.

In order to check whether a similar dsRNA would induce the same effect on Dicer-2 expression, we tested 4 μg of a 343-bp dsRNA targeting Dicer-1 mRNA (dsDcr1), following the procedure of the previous experiment. This time, as expected, mRNA levels of Dicer-1 tended to decrease 6 h after the treatment and were already significantly lower than in control (water-treated) specimens 1 day later (Fig. 2b).

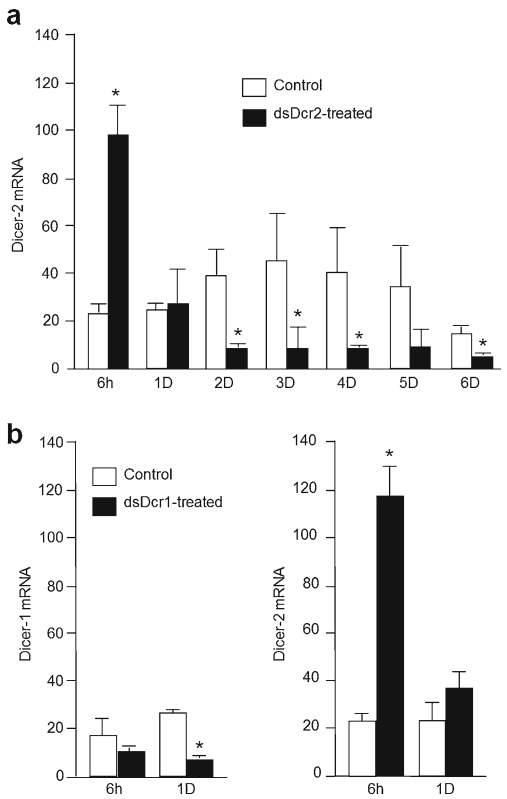

Fig. 2 Expression of Dicer-2 after exogenous application of dsDcr in *B. germanica*. **a** Expression of Dicer-2 in the whole body of the fifth nymphal instar female in controls and in specimens treated with 4 μg of dsDcr2; they were treated as freshly emerged and examined 6 h later, and then every day (*D*) during the instar. **b** Expression of Dicer-1 (*left*) and of Dicer-2 (*right*) in the whole body of the fifth nymphal instar female in controls and in specimens treated with 4 μg of dsDcr1; they were treated as freshly emerged and examined 6 h and 1 day later. Results represent the mean±SEM (n=5–9) and are expressed as copies of Dicer-1 (*left*) or Dicer-2 (*right*) mRNA per 1,000 copies of BgActin-5c mRNA; the *asterisk* indicates that differences of dsRNA-treated specimens with respect to their respective controls are statistically significant (p<0.05) according to the REST software tool (Pfaffl et al. 2002)

When Dicer-2 mRNA levels were quantified in the same samples, results were similar to those observed in the treatment with dsDcr2: a fivefold upregulation of Dicer-2 6 h after the treatment and a return to approximately normal levels 1 day later (Fig. 2b).

Super-induction of Dicer-2 expression by alien dsRNA

At this point, we wanted to test whether alien dsRNAs would induce a similar fast upregulation of Dicer-2. For this purpose, we carried out the same experiments, but this time, we used a 300-bp dsRNA sequence from *A. californica* nucleopolyhedrovirus (dsPolyH). This sequence is currently used in our laboratory as a control in RNAi experiments (Lozano and Belles 2011). The 300-bp dsPolyH was injected at a dose of 4 μg in freshly emerged fifth instar female nymphs, as in previous experiments; 6 h after the treatment, Dicer-2 mRNA levels showed a fivefold increase compared to water-treated controls (Fig. 3a), also as in previous experiments. When the dose of this dsPolyH was reduced to 0.4 μg, the induction of Dicer-2 expression was very similar, but no induction was observed when the dsPolyH dose was further reduced to 0.04 μg (Fig. 3a).

dsRNA length threshold for Dicer-2 response and specific effect of dsRNA

Once we established that a 300-bp alien dsRNA such as dsPolyH induced the upregulation of Dicer-2, we investigated the minimum size of the dsRNA that could elicit such a response. In order to address this issue, we tested dsPolyHs

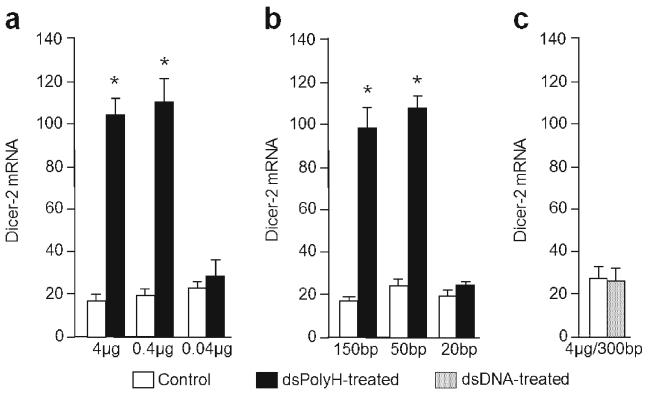

Fig. 3 Expression of Dicer-2 in specimens of *B. germanica* treated with dsRNA of PolyH (dsPolyH) or dsDNA of PolyH (dsDNA). **a** Effect of the dose of dsPolyH. **b** Effect of the length of the dsPolyH. **c** Effect of a dsDNA whose sequence corresponds to the 150 bp dsPolyH. Specimens were treated as freshly emerged fifth instar female nymphs and examined 6 h later; results represent the mean±SEM (n=8–12) and are expressed as copies of Dicer-2 mRNA per 1,000 copies of BgActin-5c mRNA; the *asterisk* indicates that differences of dsRNA-treated specimens with respect to their respective controls are statistically significant (p<0.05) according to the REST software tool (Pfaffl et al. 2002)

of different lengths (150, 50, and 20 bp) injected, respectively, at a dose of 4 μg in freshly emerged fifth instar female nymphs. Results showed that the lengths of 150 and 50 bp induced a fivefold upregulation of Dicer-2 (Fig. 3b), which was similar to that observed in the equivalent experiments using the 300-bp dsPolyH at a dose of 4 μg (Fig. 3a). Conversely, the dsPolyH of 20 bp did not elicit any significant response (Fig. 3b).

Finally, we wondered whether the property of upregulating Dicer-2 expression was specific of dsRNAs, or also dsDNAs could elicit the same effect. In order to answer this question, we tested a dsDNA of *A. californica* nucleopolyhedrovirus whose sequence corresponded to the 300-bp fragment tested as dsRNA and described above. The experiment methodology and the dose used were the same as in the dsRNA experiments, but the results (Fig. 3c) showed that dsDNA did not induce Dicer-2 expression.

An evolutionary ancient response to viral infection?

In insect RNA-based antiviral immunity, double-stranded RNAs are recognized as molecules associated with pathogens and, as a defense, are processed into siRNAs by host Dicer-2 (Ding 2010; Galiana-Arnoux et al. 2006). Moreover, striking evidence for a role of Dicer-2 as a sensor of viral infection and as a key antiviral defense element beyond the RNAi pathway has recently been demonstrated in *D. melanogaster*, where Dicer-2 mediates the induction of the antiviral gene *Vago* (Deddouche et al. 2008). Interestingly, Dicer-2 belongs to the same DExD/H-box helicase superfamily as the RIG-I-like receptors that sense viral infection and mediate interferon induction in mammals (Deddouche et al. 2008). This suggests that DExD/H-box helicase is an evolutionary old superfamily of sensors devoted to the detection of viral infections to induce defense responses by the host.

The upregulation of Dicer-2 expression after an exogenous administration of a dsRNA fits well with this sensor role. Its occurrence in *B. germanica* suggests that sensing alien RNAs might be an ancestral function of Dicer proteins, and it is even still operative in an insect that is not particularly prone to viral infections. Indeed, only densoviruses, which are single-stranded DNA viruses, are considered genuine cockroach viruses, and a densovirus has recently been characterized in *B. germanica* (Mukha et al. 2006). This reasoning leads to the prediction that the mechanism of upregulation of Dicer-2 after exogenous dsRNA administration is present in other arthropods, especially if they are prone to viral infections. Consistent with this prediction, a very recent report on the crustacean *L. vannamei* (Chen et al. 2011), which is typically prone to viral infections, shows that expression levels of Dicer-2 increase ca. sevenfold 9 h after treatment with the commercial double-stranded homopolymer Poly(C-G) (Sigma-Aldrich P4038) that is used as control in RNAi experiments. Of note, upregulation of Dicer-2 was also observed in *L. vannamei* following infection with white spot syndrome virus (Chen et al. 2011).

Finally, it is tempting to speculate that the ancestral Dicer that gave rise to Dicer-1 and Dicer-2 in the arthropod lineage would possess the dual ability to process miRNAs and siRNAs, with the latter function being related to antiviral immunity. Indeed, this dual function operates today in the single Dicer protein of *C. elegans* (Ketting et al. 2001). After the duplication, Dicer-1 might specialize in dicing miRNA precursors in the miRNA pathway, while Dicer-2 might specialize in dicing dsRNAs in the RNAi pathway. These are the respective basic functions that we observe today in those species that have been functionally studied and that possess the two Dicer genes.

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