Scavenger receptor-mediated endocytosis facilitates RNA interference in the desert locust, *Schistocerca gregaria*

N. Wynant, D. Santos, P. Van Wielendaele and J. Vanden Broeck

*Molecular Developmental Physiology and Signal Transduction, Department of Animal Physiology and Neurobiology, KU Leuven, Leuven, Belgium*

Abstract

RNA interference (RNAi) has become a widely used loss-of-function tool in eukaryotes; however, the delivery of double-stranded (ds)RNA to the target cells remains a major challenge when exploiting the RNAi-technology. In insects, the efficiency of RNAi is highly species-dependent. Yet, the mechanism of cell entry in insects has only been characterized in a cell line of the fruit fly, *Drosophila melanogaster*, a species that is well known to be poorly amenable to environmental RNAi. In the present paper, we demonstrate that silencing *vacuolar H-ATPase 16* (*vha16*) and *clathrin heavy chain* (*clath*), two components of the Clathrin-dependent endocytosis pathway, together with pharmacological inhibition of scavenger receptors with polyinosine and dextran sulphate, can significantly attenuate the highly robust RNAi response in the desert locust, *Schistocerca gregaria*.

Keywords: RNAi, double stranded RNA (dsRNA), insect, *sid1*, pattern recognition, environmental, systemic.

Introduction

RNA interference (RNAi) is a mechanism of sequence-specific gene regulation triggered by double-stranded (ds)RNA (Fire *et al.*, 1998). While RNAi is known to be involved in antiviral immunity (Waterhouse *et al.*, 2001; Zambon *et al.*, 2006), genome maintenance (Lippman & Martienssen, 2004) and regulation of endogenous gene expression (Hutvagner & Zamore, 2002), it has also become a widely used tool to knock down and analyse the function of genes in eukaryotes (Hardy *et al.*, 2010).

In addition, several recent studies have shown that RNAi may also contribute to strategies for selectively controlling agricultural pests, including a number of insect species (Baum *et al.*, 2007; Mao *et al.*, 2007; Huvenne & Smagghe, 2010); however, a major challenge in exploiting the RNAi technology remains the introduction of dsRNA into the cells (Perrimon *et al.*, 2010; Yu *et al.*, 2013).

Many organisms display RNAi after injection or oral delivery of dsRNA. This phenomenon, in which RNAi is established in tissues distant from the site of dsRNA administration, is referred to as environmental (env)RNAi. This type of RNAi requires the cellular uptake of dsRNA from the extracellular environment, but not necessarily the spreading of the RNAi-signal from cell to cell (Whangbo & Hunter, 2008). Following a screen with mutant *Caenorhabditis elegans* worms, the transmembrane channel protein Systemic RNA Interference Deficient-1 (*SID1*) was identified as a key player in the cellular uptake of dsRNA (Winston *et al.*, 2002; Duxbury *et al.*, 2005). Expression of the *Ce-sid1* sequence in *Drosophila Schneider (S)2* cells, which lack a *sid1* homologous sequence, significantly enhanced the envRNAi response (Feinberg & Hunter, 2003). In addition, homologous sequences of *sid1* in fish and mammals have been shown to mediate dsRNA import (Duxbury *et al.*, 2005; Ren *et al.*, 2011; Eihassan *et al.*, 2012). The genomes of most insects, with the exception of dipterans, also possess genes related to *Ce-sid1*. Yet, their definite functional role in RNAi remains unclear. For example, *Tribolium castaneum* and *Locusta migratoria* display a robust envRNAi response, but their *sid1* like sequences do not seem to mediate uptake of dsRNA (Bucher *et al.*, 2002; Tomoyasu *et al.*, 2008; Luo *et al.*, 2013).

Correspondence: Niels Wynant, Molecular Developmental Physiology and Signal Transduction, Department of Animal Physiology and Neurobiology, KU Leuven, Naamsestraat 59, P.O. Box 02465, B-3000 Leuven, Belgium. Tel.: +32 16323900; fax: +32 16323902; e-mail: niels.wynant@bio.kuleuven.be

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2012). By contrast, in *Apis mellifera*, the involvement of *sid1 like* in the envRNAi response was suggested, since administration of dsRNA induced an up-regulation of the *Am-sid1 like* transcript levels (Aronstein *et al*., 2006).

The best-studied insect model, *Drosophila melanogaster*, as well as many lepidopterans are poorly sensitive towards envRNAi, since injection of dsRNA into the body cavity is not an effective method to induce RNAi (Miller *et al*., 2008; Belles, 2010; Terenius *et al*., 2011). For example, in the larval stage of *D. melanogaster*, the haemocytes (and the haemocyte-like S2 cell line) seem to be the only cell type that can take up dsRNA (Miller *et al*., 2008). Yet, since *sid1 like* sequences are absent in the genomes of these flies, S2 cells must use alternative dsRNA-uptake mechanisms. Two independent functional screens demonstrated that dsRNA enters S2 cells via scavenger receptor (SR)-mediated endocytosis (Saleh *et al*., 2006; Ulvila *et al*., 2006). Saleh *et al*., (2006) suggested that Vacuolar H-ATPase 16 (Vha16) plays an important role in the redirection of exogenous dsRNA from the vesicular location of the standard endocytotic uptake route into the cytoplasm, where the RNAi-machinery is localized. In contrast to *D. melanogaster* (Belles, 2010; Miller *et al*., 2012), the desert locust, *Schistocerca gregaria*, displays a very robust envRNAi response, as virtually all tissues are sensitive towards injection of dsRNA in the body cavity and only small quantities of dsRNA are needed (Wynant *et al*., 2012). These characteristics make this species amenable to the study of the mechanisms of envRNAi in insects.

In the present study, we demonstrate that two components of Clathrin-dependent endocytosis, namely *vha16* and *clathrin heavy chain* (*clath*), and SRs play an important role in the *in vivo* envRNAi response of *S. gregaria*, while loss-of-function experiments and *in silico* analyses suggest that *Sg-SID1 like* plays no major role in the uptake of dsRNA.

### Results

#### Silencing components involved in Clathrin-dependent endocytosis

To assess the involvement of Clathrin-dependent endocytosis in the envRNAi response in *S. gregaria*, we silenced *clath* and *vha16* that mediate formation of coated vesicles and lysosomal acidification, respectively. After injection of 150 ng of the corresponding dsRNA into the abdominal body cavity (hemocoel), silencing of these two genes in the midgut was confirmed with quantitative PCR (qPCR) by comparing the transcript levels with a control group that was injected with green fluorescent protein (gfp) dsRNA (Fig. 1). The knockdown of *vha16* was assessed 6 days after the dsRNA injection; however, silencing of *clath* gene expression induced death of the injected locusts, starting from 7 days after the dsRNA administration; therefore, all experiments with *clath* dsRNA were performed 4 days after the injection. At that time, there were not yet detectable defects in the behaviour of the locusts.

#### Endocytosis-based uptake of the RNAi-signal

An ‘RNAi on RNAi’ approach was followed to investigate whether these endocytotic pathway components contribute to the envRNAi response in the RNAi-sensitive midgut tissue. First, we silenced *vha16* by injection of 150 ng of its transcript-specific dsRNA. Six days later, a second injection was performed with 150 ng of *alpha-tubulin 1a* (*tubu*) dsRNA. The potency of the *tubu* knockdown was determined 6 days after injection of 150 ng of double-stranded (ds)RNA of *vha16* and 4 days after injection of 150 ng of *clath* dsRNA. The transcript levels were compared with locusts injected with 150 ng of dsRNA of *gfp* (**: *P* < 0.005; *n* ≥ 6).
In addition, the RNAi response was assessed in midgut tissues, as this tissue was previously shown to be highly sensitive towards envRNAi (Wynant et al., 2012). As a control experiment, locusts were injected with dsRNA for gfp, and 6 days later, they were injected with tubu dsRNA. A second control group that was treated twice with gfp dsRNA was also taken into account. Because in this group the tubu gene expression was not silenced, these locusts were used to determine the normal physiological tubu expression levels. Our results show that knocking down the tubu transcript level was significantly less potent when vha16 was down-regulated, i.e., in comparison with the first control group (Fig. 2A). Still, it remained possible that silencing vha16 had some effect on the tubu transcript levels, rather than impairing the uptake of RNAi; therefore, we also silenced vha16 and measured the tubu transcript levels. The tubu expression levels remained indistinguishable upon silencing vha16 from those in the gfp dsRNA-injected control conditions (Fig. 2B). Second, we assessed whether silencing clath could also impair the envRNAi response. To ascertain that the observations were not specific for silencing tubu, we chose a different marker gene, namely glyceraldehyde 3-phosphate dehydrogenase (gapdh), to determine the knockdown potency. The knockdown of gapdh was significantly less robust when clath was down-regulated (Fig. 2C), while silencing clath had no direct effect on the gapdh transcript levels (Fig. 2D).

Scavenger receptors are important for envRNAi

Since no sequence information of S. gregaria SRs was available in the S. gregaria expressed sequence tag database, we used well-known inhibitors of the SR family, namely polyinosine [poly(I)] and dextran sulphate (DS) (Abrams et al., 1992; Pearson et al., 1993, 1995; Krieger & Herz, 1994; Whitman et al., 2000). Regarding the fact that an adult desert locust possesses, on average, 200 μl haemolymph (Lee, 1961), and that administration of 400 μg/ml of poly(I) or DS to the medium of Drosophila L2 cells could inhibit >85% of the SR activity (Abrams et al., 1992), 0.1 mg of the inhibitors was injected into the body...
expression levels were determined 1 h after the injection (CS, respectively, had no direct effect on the...sulphate (CS) and 1 h later injected with 150 ng of dsRNA of (poly[C], polyadenosine (poly[A]), dextran sulphate (DS) or chondroitin...in many other insect species. Since these sequences...transcript levels. Moreover, sequence comparison of the deduced amino acid sequence of Sg-sid1 like showed higher sequence similarity with ChUP1 (previously known as TAG-130) than with SID1 of C. elegans. Ce-ChUP-1 was also predicted to have a highly similar topological structure to the Sg-SID1 like protein (Supplementary data, Fig. S2) and comparison of their N-terminal extracellular domain revealed regions with high conservation with Ce-ChUP1, but not with Ce-SID1 (Fig. SC).

Discussion

By silencing two distinct components of the Clathrin-dependent endocytosis machinery and by pharmacological inhibition of SRs, we have demonstrated that SR-mediated uptake contributes to the robust RNAi response in the desert locust, S. gregaria. In contrast, knocking down Sg-sid1 like generated no clear effects on RNAi efficiency. Moreover, sequence comparison revealed higher sequence similarity with Ce-ChUP1 than with Ce-SID1. Yet, a study by Tomoyasu et al. (2008) has demonstrated that Ce-ChUP1 is not involved in dsRNA uptake in C. elegans, but it mediates the uptake of dietary cholesterol (Valdes et al., 2012). Whether Sg-sid1 like is also involved in cholesterol transport rather than dsRNA uptake remains to be determined, but it should be noted that the effect of Sg-sid1 like was not assessed at the functional protein level so it remains possible that the protein knockdown was too modest to generate clear phenotypic effects. Sid1 like sequences have been identified in many other insect species. Since these sequences also display higher sequence similarity with Ce-ChUP1, it...
appears to be a misconception that these sequences are true homologues of Ce-SID1.

Although *S. gregaria* and *C. elegans* display a robust envRNAi response, these data suggest on the one hand that *S. gregaria* uses different dsRNA uptake mechanisms. On the other hand, it might seem surprising that S2 cells, an envRNAi-sensitive cell line derived from the largely envRNAi-insensitive *D. melanogaster*, use the same dsRNA entry route (Saleh *et al.*, 2006; Ulvila *et al.*, 2006); however, the tissue specificity and expression levels of components involved in this uptake mechanism may determine the difference in success of the dsRNA transport. As pointed out by several recent reports, the efficiency of the cell-autonomous RNAi machinery may also be a determinant factor for the potency of the knockdown (Swevers *et al.*, 2011; Garbutt & Reynolds, 2012). The observation that the envRNAi response was only partially inhibited may still suggest the presence of alternative parallel dsRNA-uptake mechanisms. Nevertheless, we have previously demonstrated that injection of pg amounts of dsRNA per mg tissue can already evoke a clearly detectable RNAi knockdown in *S. gregaria* (Wynant *et al.*, 2012), therefore, one can expect that residual cell entry of dsRNA, attributable to incomplete silencing or inhibition of the uptake mechanisms, would still result in activation of the RNAi response. This might indeed have been the case in our experiments, keeping in mind the technical limitations of the applied procedures. First, competitively blocking the SRs with poly(I) or DS may have been reversible and incomplete. Second, a knockdown (established using dsRNA injections in the desert locust) does not generate loss-of-function effects to the same degree as a complete knockout; therefore, to date, it remains uncertain whether additional parallel cell entry routes are also functional in the desert locust.

**Figure 4.** The predicted topologic structure of (A) *Sg*-SID1 like and (B) Ce-SID1 using PHOBIUS software.
Besides the ability of nematode cells to efficiently take up dsRNA from their extracellular environment via SID1, studies have shown that the RNAi signal is also amplified by RNA-dependent-RNA-polymerases (RdRPs) in these cells (Smardon et al., 2000; Sijen et al., 2001), ensuring high potency and prolonged effectiveness of RNAi. Whereas ticks also possess RdRPs, they seem to have lost RdRP-like interferon response) (Limmon et al., 2005; Pluddemann et al., 2007) and dsRNA uptake in the desert locust remains to be demonstrated. Yet, since SR-mediated endocytosis is a well-described uptake route for polyanionic ligands (Taylor et al., 2005; Pluddemann et al., 2007) and dsRNA uptake via this uptake mechanism has been directly demonstrated in mammalian cell lines (to activate the interferon response) (Limmon et al., 2008; Dieudonne et al., 2012). Nevertheless, this suggests conservation of SR-mediated dsRNA-uptake. Furthermore, several studies have highlighted the importance of endocytosis-based spreading of the RNAi-signal in the nematode, C. elegans (Hinas et al., 2012; Jose et al., 2012).

Direct participation of SR-mediated endocytosis in dsRNA uptake in the desert locust remains to be demonstrated. Yet, since SR-mediated endocytosis is a well-described uptake route for polyanionic ligands (Taylor et al., 2005; Pluddemann et al., 2007) and dsRNA uptake via this uptake mechanism has been directly demonstrated in mammalian cell lines (to activate the interferon response) (Limmon et al., 2008; Dieudonne et al., 2012) and in Drosophila S2 cells (Saleh et al., 2006; Uvlila et al., 2006), a role in in vivo dsRNA uptake in S. gregaria might seem plausible.

The use of the RNAi technology as a research tool and as an alternative insect control method is currently hindered because of the poor sensitivity towards envRNAi of many (economically important) insect species, such as D. melanogaster, Bombbyx mori, Manduca sexta and Spodoptera litura (Miller et al., 2008; Iga & Smagghe, 2010; Terenius et al., 2011). Moreover, this has led to the development of (repositories of) RNAi transgenes for long dsRNA induces interferon responses, rather than being an RNAi signal (Limmon et al., 2008; Dieudonne et al., 2012). Nevertheless, this suggests conservation of SR-mediated dsRNA-uptake. Furthermore, several studies have highlighted the importance of endocytosis-based spreading of the RNAi-signal in the nematode, C. elegans (Hinas et al., 2012; Jose et al., 2012).

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model organisms, such as D. melanogaster (Dietz et al., 2007), which is in contrast to the ease of applying RNAi in envRNAi-sensitive insect species, where injection (and in some cases even oral delivery) of dsRNA induces gene silencing throughout the body (Belles, 2010; Huvenne & Smagghe, 2010). In this context, the knowledge obtained in the present paper may be employed to improve gene-silencing techniques in model organisms with reduced envRNAi sensitivity. For instance, this could be achieved by expression of dsRNA-sensitive SRs in Drosophila cells or tissues that are refractory to import dsRNA. An alternative approach may be found in improving the sensitivity of the cell-autonomous RNAi-response by up-regulation of core RNAi-components. In conclusion, we showed that SR-mediated endocytosis contributes to the envRNAi-response in the desert locust. As the present study is the first report on an in vivo dsRNA transport mechanism in an envRNAi-sensitive insect species, it contributes to a better understanding of the mechanisms of envRNAi in insects and can possibly support the search for the improvement of envRNAi efficiency in other species.

Experimental procedures

Rearing of the desert locust, Schistocerca gregaria

Gregarious S. gregaria were reared under crowded conditions with controlled temperature (32 ± 1°C), light (13 h photoperiod) and relative humidity (40–60%). They were fed daily with fresh cabbage and dried oat flakes. Adult locusts were developmentally synchronized by transferring them to a different cage directly after their final moult.

Sequence information

Transcript sequence information of S. gregaria tubu DHO851397, gapdh DHO851387, vha16 KJ135006 and clath KJ135005 was retrieved from the annotated S. gregaria expressed sequence tag database (Badisco et al., 2011). To verify the sequences, the DNA fragments were cloned into the pCR®4-TOPO® vector by means of the TOPO TA Cloning® Kit for Sequencing (Life Technologies Co., Foster City, CA, USA). The fragments were subsequently cloned in a cPCR®-TOPO® vector using the TOPO TA Cloning® Kit for Sequencing (Life Technologies Co.) and sequenced according to the ABI PRISM BigDye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems).

Synthesis of dsRNA

Double stranded RNAs for clath (561 bp), vha16 (453 bp), sid1 like (250 bp), tubu (545 bp), gapdh (447 bp) and gfp (589 bp) were synthesized using the MEGAscript RNAi kit (Ambion, Austin, TX, USA). A DNA template flanked by two T7 promoter sequences was synthesized for production of clath, vha16, sid1 like, tubu and gapdh dsRNA; therefore, a PCR reaction was performed with cDNA of adult S. gregaria midgut tissue, gene-specific primers containing a T7 promoter sequence at the 5’-end (Sigma-Aldrich Co.), and REDTaq mix (Sigma-Aldrich Co.) as a source of DNA Taq polymerase, dNTPs and PCR buffer. All PCR primers are shown in the supplementary data (Fig. S3). The amplification products were subsequently analysed by 1% agarose gel electrophoresis and visualized with UV light. The PCR product was used directly as template for production of dsRNA. Synthesis of gfp dsRNA was performed using a TOPO 4.1 sequencing vector (Life Technologies Co.) containing a gfp transcript sequence as DNA template, as described in Wynant et al., 2012. After the production of dsRNA, the remaining DNA and ssRNA was removed by nuclease treatment, and proteins and mono/oligonucleotides were removed by solid-phase adsorption purification, according to the manufacturers’ specifications (Ambion). The dsRNA-concentration was determined by means of a Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and the integrity of the dsRNA was assessed by gel electrophoresis using a 1% agarose gel. The dsRNA was stored at −20°C until further use.

Injection and tissue collection of adult locusts

Locusts were each injected with 10 μl of dsclath, dsvha16, ds sid1 like, dstubu, dsgapdh or dsgfp or with poly(I) (Sigma-Aldrich Co.), poly(A) (Sigma-Aldrich Co.), DS (Sigma-Aldrich Co.) or CS (Sigma-Aldrich Co.) containing solution that was diluted in 0.746 g KCl; 0.407 g MgCl2; 0.336 g NaHCO3; 30.807 g sucrose; 1.892 g trehalose; pH 7.2). Midgut tissue was collected and cleaned by microdissection in S. gregaria Ringer solution under a binocular microscope, and immediately transferred to liquid nitrogen to prevent RNA degradation. Samples were stored at −80°C until further processing.

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**RNA extraction and cDNA synthesis**

The Lipid tissue extraction kit (Qiagen, Valencia, CA, USA) was used to extract RNA, following the manufacturer’s specifications. Quality and concentration of the extracted RNA were assessed using a Nanodrop spectrophotometer. Next, equal amounts of RNA were used as template to produce cDNA. The cDNA synthesis was performed using the PrimeScript™ First-strand cDNA Synthesis Kit (TaKaRa, Kyoto, Japan), as described in the corresponding protocol. Finally, the cDNA solution was 15-times diluted with MilliQ water (Millipore, Billerica, MA, USA). The resulting cDNA-samples were analysed immediately or stored at −20 °C until further usage.

**Quantitative real-time PCR**

Primer express software (Applied Biosystems) was used to design qPCR primers. The corresponding primer sequences are shown as supplementary data in Fig. S4. The primers were validated, the most stable reference genes were selected and the qPCR reaction was performed as described in Wynant et al. (2012). To correct for the sample-to-sample variation, the relative transcript levels were normalized against ubiquitin conjugating enzyme 10 and elongation factor 1a. The data were further normalized against a calibrator cDNA sample to account for variation in the PCR efficiency in different PCR runs. In every experiment, no-template controls were included to check for contamination. The PCR reaction was performed and analysed in a 96-well plate and by the StepOne System (ABI prism, Applied Biosystems). Since the efficiency of the different primers was the same, the relative transcript quantity was calculated according to the delta–delta Ct method. All data were statistically analysed using non-parametric statistics in GRAPHPAD PRISM 5 (GraphPad).

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**References**


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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:
Figure S1. Six days after the injection of sid1 like or gfp dsRNA, the tubu transcript level was determined using quantitative PCR ($P < 0.014$, $n \geq 7$).

Figure S2. The predicted topological structure of Ce-ChUP1 using Phoebus software.

Figure S3. Sequences of the primers used to make the template for dsRNA production. The T7-promotor site is indicated in bold.

Figure S4. Sequences of the quantitative PCR primers.