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Clathrin-dependent endocytosis plays a predominant role in cellular uptake of double-stranded RNA in the red flour beetle

Da Xiao [a, b], Xiwu Gao [b], Jiaping Xu [a, c], Xiao Liang [a], Qingqing Li [a, d], Jianxiu Yao [a], Kun Yan Zhu [a, *]

[a] Department of Entomology, Kansas State University, Manhattan, KS 66506, USA
[b] Department of Entomology, China Agricultural University, Beijing 100094, China
[c] School of Life Sciences, Anhui Agricultural University, Hefei, Anhui 230036, China
[d] College of Life Sciences, Yunnan Normal University, Kunming, Yunnan 650092, China

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ABSTRACT

RNA interference (RNAi) is a highly conserved gene regulatory mechanism in eukaryotic organisms; however, an understanding of mechanisms of cellular uptake of double-stranded RNA (dsRNA) in different organisms remains elusive. By using pharmacological inhibitors of different endocytic pathways in conjunction with RNAi of a marker gene (*lethal giant larvae*, *TcLgl*) in the red flour beetle (*Tribolium castaneum*), we demonstrated that two inhibitors (chlorpromazine and bafilomycin-A1) of clathrin-dependent endocytosis can nearly abolish or significantly diminish RNAi of *TcLgl*, whereas methyl-β-cyclodextrin and cytochalasin-D, known to inhibit other endocytic pathways, showed no effect on RNAi of *TcLgl*. By using Cy3-labeled *TcLgl* dsRNA, we observed significantly reduced cellular uptake of *TcLgl* dsRNA in midgut cells after larvae were injected with each of the two clathrin-dependent endocytosis inhibitors. By using an "RNAi of RNAi" strategy, we further demonstrated that suppression of each transcript of the four key genes encoding clathrin heavy chain (*TcChc*), clathrin coat assembly protein AP50 (*TcAP50*), vacuolar (H^+)-ATPase subunit H (*TcVhaSFD*) and a ras-related protein (*TcRab7*) in clathrin-dependent endocytosis by RNAi can significantly impair RNAi of *TcLgl*. These results support our conclusion that clathrin-dependent endocytosis is a major mechanism in cellular uptake of dsRNA in *T. castaneum*. Our study also provides new insights into improving RNAi efficiency by enhancing dsRNA endosomal release.

1. Introduction

RNA interference (RNAi) is a highly conserved post-transcriptional gene regulatory mechanism in eukaryotic organisms, including fungi, plants, insects and mammals (Fire et al., 1998; Mello and Conte, 2004; Bellés, 2010; Zhuang and Hunter, 2011). However, the cellular uptake mechanism of exogenous double-stranded RNA (dsRNA) does not appear to be highly conserved and remains undefined in different organisms. Two pathways for exogenous dsRNA uptake have been identified or implicated, which include passive uptake via a transmembrane channel protein known as systemic RNA interference deficient-1 (SID-1) encoded by *sid-1* in the nematode (*Caenorhabditis elegans*) (Winston et al., 2002), the honey bee (*Apis mellifera*) (Aronstein et al., 2006), and the fish (*Siniperca chuatsi*) cells (Ren et al., 2011); and endocytosis-mediated pathway in the fruit fly (*Drosophila melanogaster*) S2 cells (Ulvila et al., 2006; Saleh et al., 2006), the desert locust (*Schistocera gregaria*) (Wynant et al., 2014), and the predatory mite (*Metaseiulus occidentalis*) (Wu and Hoy, 2014).

Although a putative transmembrane protein encoded by *AmSid-1* was found to play an essential role in dsRNA uptake in the honey bee (Aronstein et al., 2006), several studies have shown that SID-1 is unlikely to play a major role in cellular uptake of dsRNA in other insects including the red flour beetle (*Tribolium castaneum*) (Tomoyasu et al., 2008), the migratory locust (*Locusta migratoria*) (Luo et al., 2012), and the desert locust (Wynant et al., 2014). Because SID-1-dependent dsRNA uptake does not seem to be common and conserved in insects, many insects must possess additional or different genes with similar functions, or possibly

* Corresponding author. Tel.: +1 785 532 4721; fax: +1 785 532 6232.
E-mail address: kzhu@ksu.edu (K.Y. Zhu).

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even different mechanisms in cellular uptake of dsRNA (Tomoyasu et al., 2008; Zhang et al., 2010).

Ulvila et al. (2006) analyzed 2000 dsRNA fragments from a cDNA library of *D. melanogaster* S2 cells for their protective effect from lethality induced by RNAi against *Ubi-p63E*, an essential gene encoding an ubiquitin for cell viability. They identified four genes, one of which encodes clathrin heavy chain (*Chc*), an important component of clathrin-dependent endocytosis. When *Chc* transcript in S2 cells is depleted by RNAi, it protects S2 cells from the lethality induced by the *Ubi-p63E* dsRNA treatment, suggesting that dsRNA molecules are internalized by clathrin-dependent endocytosis. On the other hand, Saleh et al. (2006) screened a dsRNA library of *D. melanogaster* S2 cells and identified 23 genes likely to be involved in endocytic pathway and required for cellular uptake of dsRNA by S2 cells. Some of these genes have been known to be directly and/or indirectly involved in endocytosis as they encode proteins of the vesicle mediated transport, conserved oligomeric Golgi complex family, cytoskeleton organization and protein transport.

Results from previous studies suggest that most insects are likely to rely on different mechanisms rather than *C. elegans* SID-1-dependent dsRNA uptake pathway (Tomoyasu et al., 2008; Zhang et al., 2010), and endocytosis plays an important role in dsRNA uptake in *D. melanogaster* S2 cells and probably in many insect species such as the desert locust (Wynant et al., 2014). However, the exact mechanism of cellular dsRNA uptake in different insects remains unclear. In this study, we took the advantage of *T. castaneum* for its robust RNAi to examine: 1) the effect of pharmacological inhibitors of different endocytic pathways on RNAi of a marker gene (*TcLgl*, *lethal giant larvae*); 2) the effect of selective inhibitors of clathrin-dependent endocytosis on cellular uptake of Cy3-labeled *TcLgl* dsRNA in larval midgut; and 3) effect of RNAi targeting each of four key genes in clathrin-dependent endocytosis on RNAi of *TcLgl* (i.e., a "RNAi of RNAi" strategy). Our studies provided strong evidence that clathrin-dependent endocytosis plays an essential role in cellular uptake of dsRNA in *T. castaneum* larvae.

2. Materials and methods

2.1. Insect culture

The Georgia-1 (GA-1) strain of *T. castaneum* was reared on whole-wheat flour containing 5% (by weight) of brewers' yeast at 30 °C and 65% RH in growth chamber at Kansas State University (Manhattan, KS, USA).

2.2. Subcloning and sequencing of TcChc

Total RNA was isolated from the insects by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase I (Fermentas, Glen Burnie, MD, USA) to remove possible genomic DNA contamination. First-strand cDNA was synthesized from 1.0 μg total RNA by using First Strand cDNA Synthesis Kit (Fermentas). Specific primers designed based on the predicted gene sequences were used to obtain full-length cDNA by reverse transcription PCR (RT-PCR) (Table 1). After PCR products were purified and ligated into pGEM-T Easy Vector (Invitrogen), the ligation mixture was used to transform DH5α competent cells. Plasmid DNA was purified and sequenced by the KSU DNA Sequencing and Genotyping Facility (Manhattan, KS, USA).

2.3. Procedures of RNAi

dsRNA was synthesized using MEGAscript® RNAi Kit (Ambion, Austin, TX). After each larva was injected in the hemocoel with 100 nl of dsRNA (100 ng/larva) by using Nanoject II injector (Drummond Scientific, Broomall, PA, USA), the larvae were reared under standard conditions. RNAi was performed with three biological replicates (each with at least 40 larvae) for each control and treatment.

2.4. Determination of gene transcript level

Developmental stage-dependent expression profiles of *TcChc* were analyzed in all four life stages at 20 developmental time points, including embryos (1, 2, 3, and 4-day eggs), larvae (1, 5, 10, 15, and 20-day), pupae (1, 2, 3, 4, 5, and 6-day) and adults (1, 5, 10, 15, and 20-day). In each replicate, 60 eggs, 5 larvae, 5 pupae, or 5 adults were pooled as a biological sample for 20 developmental time points. Because our preliminary studies showed a large peak of *TcChc* transcript during the mid-pupal stage (i.e., 2, 3 and 4-day), we dissected four tissues, including the gut (midgut and hindgut), Malpighian tubule, fat bodies and carcass (the remaining body after the brain, ganglia, gut and fat bodies removed), from 3-day old pupae for profiling tissue-dependent expression of *TcChc*. In each replicate, Malpighian tubules dissected from 60 pupae were pooled or each of the three remaining tissues dissected from 30 pupae were pooled as a biological sample.

Total RNA was isolated from each sample by using TRIzol reagent (Invitrogen) and treated with DNase I (Fermentas). First-strand cDNA was synthesized from 1.0 μg total RNA by using First Strand cDNA Synthesis Kit (Fermentas). Reverse transcription quantitative PCR (RT-qPCR) was performed by using SYBR Green in iCycler iQTM multi-color real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) according to Xiao et al. (2014). The specificity of each reaction was evaluated based on the melting temperatures of the PCR products. RT-qPCR analysis of each developmental time point or tissue type was performed with three biological replicates (each with two technical replicates). The gene-specific primers (Table 1) were designed by using the Beacon 7.0 software. The relative expression level of each gene was normalized to ribosomal protein S3 (*TcRps3*) (Xiao et al., 2014) and calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.5. Evaluation of endocytosis inhibitors' effect on RNAi

Four endocytosis inhibitors, including chlorpromazine (CPZ), methyl-β-cyclodextrin (MβCD), bafilomycin-A1 (BafA) and cytochalasin-D (CCD), were purchased from Sigma–Aldrich (St Louis, MO, USA). CPZ and MβCD were diluted with PBS buffer, whereas BafA and CCD were diluted with 20% dimethylsulfoxide (DMSO). Initial results showed that the injection of 100 nl per larva of CPZ, MβCD, BafA and CCD at 8.0, 13.0, 0.05 and 0.50 μg/μl (i.e., 0.8, 1.3, 0.005 and 0.05 μg/larva), respectively, did not cause larval mortality and marker gene (*TcLgl*) expression change as evaluated by RT-qPCR (Supplementary Fig. 1). We used these doses and two lower doses (5-fold serial dilutions) of each inhibitor (i.e., 3 doses for each inhibitor) to examine the effect of each inhibitor on RNAi of *TcLgl*. Specifically, 100 nl of *GFP* dsRNA (ds*GFP*, 100 ng) in inhibitor solvent, *TcLgl* dsRNA (ds*TcLgl*, 100 ng) in inhibitor solvent, or ds*TcLgl* (100 ng) containing each of the four inhibitors was injected into the hemocoel of a 16-day-old larva. Each control or treatment for each inhibitor dose and for each time point consisted of three replicates (each with at least 40 larvae). The relative transcript level of *TcLgl* was determined by RT-qPCR as described above at 24, 48 and 72 h after the injection.