Double-stranded RNA Is Internalized by Scavenger Receptor-mediated Endocytosis in Drosophila S2 Cells* beloved

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Many organisms mount specific defense responses to silence invading nucleic acid sequences before these sequences integrate into the host genome and disturb cellular processes. At the core of these sequence-directed immunity mechanisms is dsRNA,3 which becomes processed and causes gene silencing, referred to as RNA interference (RNAi).4 In its defense function, RNAi guides endogenous and regulatory processes and is used as a research tool to suppress the expression of cellular genes in numerous model organisms (2).

Although species variations exist, the silencing mechanisms in plants, fungi, worms, insects, and mammals share common features and conserved genes. Drosophila S2 cells are widely used to carry out large scale functional screens (3–5) because dsRNA fragments (usually about 500–700 bp) can be added directly to the cell culture medium, “soaking” the cells rather than transfecting them (6). This makes silencing in these cells easy and efficient, in contrast to mammalian cells that require small interfering RNAs (siRNAs) to be delivered by transfection. In contrast to Caenorhabditis elegans cells, which internalize dsRNA by the channel-forming transmembrane protein SID-1 (7), the mechanism of dsRNA uptake into Drosophila cells is unknown.

In the cytosol, dsRNA fragments are processed into short 21–23-nucleotide dsRNA duplexes (siRNAs) by a dsRNA-specific RNAase-III-type endonuclease called Dicer-2 (8–10). Dicer-2 is stably complexed with the dsRNA binding domain-containing protein R2D2 binding to siRNA and thereby, facilitating siRNA loading onto RNA-induced silencing complexes (RISC) (11). RISC contain a member of the Argonaute (Ago) protein family Ago-2, which has been shown to mediate siRNA-directed mRNA cleavage (12) and degradation of the passenger strand in the siRNA duplex (13, 14). Although Dicer-2, R2D2, and Ago-2 can constitute “minimal” RISC under certain circumstances (15), many other proteins have been suggested to be involved in RNA silencing in vivo (16–18). In particular, it has been postulated that an RNA helicase is required for loading of the RISC with the guide strand of the siRNA duplex. In Drosophila, three putative RNA helicases (Armitage, Spindle E, and Dmp68) have been implicated in RNAi (16–19).

To gain a better understanding of the mechanisms of dsRNA uptake, transfer, and processing in S2 cells, we carried out an RNAi-based screen to identify gene products required for RNAi. 2,000 dsRNAs were analyzed for their protective effect from lethality induced by RNAi against an essential gene for cell viability, the Drosophila homologue of ubiquitin (Ubi-p63E) (3, 4). In addition, the mechanism and two receptors for dsRNA internalization in S2 cells were identified.

EXPERIMENTAL PROCEDURES

Synthesis of the dsRNAs—A cDNA library derived from S2 cells (20) was used as a source for templates for RNA synthesis essentially as described earlier (3). Targeted dsRNA treatments were carried out as described (21) using primers presented in a supplemental table. GFP dsRNA was produced using pMT/BiP/VS-His/GFP plasmid (Invitrogen) as a template. FITC-labeled GFP dsRNA was synthesized using a T7 MegaScript kit according to the manufacturer’s instructions, except that half of the UTP was replaced with 1.5 mM FITC-UTP (Enzo).

Ubi-p63E Survival Assay—S2 cells were grown as described (20). dsRNAs corresponding to indicated or random genes were introduced
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into S2 cells by soaking. On a 24-well plate, a total of 5×10^5 cells/well were treated with 10 μg of dsRNA. After 72 h, one-sixth of the cells treated with the first dsRNA was transferred into a 48-well plate, and Ubi-p63E dsRNA (5 μg) was introduced. 72 h later, the ability of the S2 cells to phagocytose FITC-labeled Escherichia coli was measured using flow cytometry (3).

Luciferase Reporter Assay—S2 cells were transferred to 24-well plates, and 5 μg of dsRNA was added. After 48 h, the cells were transfected with 0.5 μg of constitutively active form of Toll (Toll10b) together with 0.5 μg of Drosomycin-luciferase firefly reporter, 0.1 μg of control plasmid encoding Renilla luciferase (Promega), and either 0.5 μg of Myd88 dsRNA or 0.5 μg of GFP dsRNA using FuGENE transfection reagent (Roche Applied Science). 96 h after transfections, cells were lysed, and luciferase activity was measured. The ratio of firefly luciferase to Renilla luciferase activities was calculated, and the values were normalized to the values of GFP dsRNA-treated controls.

Endocytosis Assay—For the S2 cells, 2×10^5 cells/well were seeded into 48-well plates for flow cytometry. After 72 h, 7–20 μg/ml FITC-labeled dsRNA (500 bp) or 4 μg/ml Alexa-acetylated low density lipoprotein (AcLDL) (Molecular Probes) was added to the growth medium and incubated for 20 min. The extracellular fluorescence was quenched by adding trypsin blue just prior to the actual measurement. Thereafter, the cells were collected and analyzed by flow cytometry. For the Chinese hamster ovary (CHO) cells, 10^5 cells/well were seeded into 6-well plates for flow cytometry. 36–48 h later, 2 μg/ml Alexa-AcLDL or 7.5 μg/ml FITC-dsRNA (500 bp) was added to the growth medium and incubated for 2 h. Thereafter, the cells were washed with 1×PBS, detached by trypsinization, pelleted by centrifugation, resuspended in ice-cold 1×PBS, and analyzed by fluorescence-activated cell sorting.

Confocal and Fluorescence Microscopy—10^6 S2 cells/well were seeded into two chamber slides (Nunc) and, when indicated, treated with a total of 20 μg of indicated dsRNA. 72 h later, cells were allowed to internalize FITC-labeled dsRNA (500 bp) or 4 μg/ml Alexa-AcLDL for 30 min at 26 °C. Cells were then washed with 1×PBS and thereafter fixed with 4% paraformaldehyde. Fixed cells were washed again with 1×PBS, and the cell nuclei were stained for 20 min with 1 μg/ml 4′,6-diamidino-2-phenylindole. After the staining, slides were washed twice with 1×PBS and once with distilled H_2O and mounted on Aquamount. For the CHO cells, 10^5 cells/well were seeded into 6-well plates and acid-washed onto 22×22-mm coverslips for microscopy. 36–48 h later, 2 μg/ml Alexa488-AcLDL or 7.5 μg/ml FITC-dsRNA was added to the growth medium and incubated for 4 h. Cells were washed with 1×PBS and fixed for 20 min with 3% formaldehyde. Fixed coverslips were washed with 1×PBS, unreacted aldehyde groups were saturated with 50 mM ammonium chloride in 1×PBS, and coverslips were mounted on 15 μl of moviol. Pictures were taken on an inverted Nikon TE2000-U epi-fluorescence microscope equipped with a cooled CCD camera (Hamatsu Orca ER) using a 60× Plan-Apochromat oil objective. Images were acquired and enhanced using Openlab software (Improvision).

Immunoelectron Microscopy—2×10^6 cells were seeded on 10-cm dishes, and 10 μg/ml dsRNA was added. After 72 h, cells were allowed to internalize FITC-dsRNA (8.5 μg/ml) for 0, 5, or 60 min at 26 °C. The cells were fixed in 4% paraformaldehyde in 0.2 M phosphate buffer with 2.5% sucrose for 30 min. The fixed cells were pelleted and immersed in 2% agarose in 1×PBS, after which the blocks were immersed in 2.3 M sucrose in 1×PBS and frozen in liquid nitrogen. For the immunolabeling, thin sections were first incubated in 0.05 M glycine in 1×PBS followed by incubation in 5% BSA with 0.1% cold water fish skin gelatin (Aurion) in 1×PBS. Antibodies and the gold conjugate were diluted in 0.1% BSA-C (Aurion) in 1×PBS. All washings were performed in 0.1% BSA-C in 1×PBS. The sections were incubated with anti-FITC antibody (Zymed Laboratories Inc.) (1:500 dilution) followed by protein A-gold complex (10 nm) for 30 min. The sections were embedded in methylcellulose and examined in Philips CM100 transmission electron microscope (FEI Co.). Images were captured with a CCD camera equipped with TCL-EM-Menu version 3 from Tietz Video and Image Processing Systems GmbH (Gaunting, Germany).

RESULTS

To identify components of the RNAi machinery in S2 cells, we devised an assay based on the observation that RNAi directed against the essential gene Ubi-p63E kills S2 cells (4) and abolishes their ability to phagocytose fluorescent particles (3). This observation prompted us to use phagocytosis as a sensitive readout for the RNAi-mediated rescue from the lethal effects of Ubi-p63E dsRNA. When the synthesis of an essential component of RNAi machinery is prevented by RNAi, the cells are protected from Ubi-p63E RNAi-mediated lethality, and their ability
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**FIGURE 2.** Belle, Pros45, and Ago-2 are required for intracellular silencing mechanisms. A. Belle and Pros45 act downstream of dsRNA internalization. S2 cells were treated with the indicated dsRNAs (10 μg/ml) for 48 h. Thereafter, dsRNA treated cells were transfected with Drosomycin-luciferase reporter plasmid, a plasmid encoding constitutively active form of Toll (Toll10b), a control plasmid encoding Renilla luciferase, and dsRNA directed against Myd88. Luciferase activities were monitored 96 h after transfection. Data are shown as means ± S.D. of at least five independent dsRNA treatments. B. Disruption of the endocytic pathway by bafilomycin A1 prevents dsRNA-mediated gene silencing in S2 cells. S2 cells were transfected with Toll10b, Drosomycin-luciferase, and Renilla luciferase as in A. Thereafter, cells were washed, and 100 nM bafilomycin A1 was added to culture medium. After 30 min, Myd88 dsRNA was added by soaking. The luciferase activity was measured after 72 h. *, p < 0.01; **, p < 0.001.

To phagocytose is preserved. As shown in Fig. 1A, S2 cells lose the ability to phagocytose E. coli within 48 h after addition of Ubi-p63E dsRNA into the cell culture medium. Pretreatment of cells with Dicer-2 dsRNAs decreased the lethal effect of Ubi-p63E dsRNA in a concentration-dependent way (Fig. 1, A and B).

We screened 2,000 dsRNA fragments generated from random S2 cell-derived cDNA clones (3). Most treatments had little or no effect, whereas altogether four RNAi treatments rescued from RNAi-induced lethality to an extent comparable to RNAi (which was used as a positive control) (Fig. 1C). These were dsRNAs targeting the endonuclease Ago-2, a well established component of the RNAi machinery in *Drosophila* (12, 22, 23), and three genes that had not been implicated in RNAi in *Drosophila* previously: Belle, a recently characterized DEAD-box RNA helicase; Pros45, the regulatory subunit 8 of the 26 S proteasome; and clathrin heavy chain (Chc), a component of the endocytic machinery.

The finding that Chc, a component of the endocytic machinery, is required for RNAi in S2 cells suggests that it plays a role in the uptake of dsRNA, whereas Ago-2, belle, and Pros45 are more likely to act at later steps in the silencing process. To confirm this prediction, we used an assay that bypasses dsRNA internalization by transfection. In this assay, transcriptional activation of a reporter gene (*Drosomycin-luciferase*) by a constitutively active form of the cell surface receptor Toll (Toll10b) is prevented by RNAi targeting, a downstream component of the Toll signaling pathway (*Myd88*). When an essential component of the RNAi machinery is blocked by RNAi, silencing of the Toll pathway is prevented, and the reporter gene is transcribed. As shown in Fig. 2A, RNAi targeting Ago-2, belle, and Pros45 (as well as a Dicer-2 control) interfered with *Myd88* dsRNA-mediated silencing of Toll pathway signaling as measured by luciferase activity. Chc dsRNA did not affect silencing of reporter gene expression. These data confirm on the one hand the involvement of Belle and Pros45 in RNAi silencing and indicate on the other hand that Chc acts upstream of intracellular silencing events.

To confirm the role of endocytic pathway in RNAi, we used a variation of the reporter gene assay as described above. We pretreated S2 cells with bafilomycin A1, an inhibitor of vacuolar type H⁺-ATPase required for endosome maturation, before soaking the cells with dsRNA directed against *Myd88*. As shown in Fig. 2B, pretreatment with bafilomycin A1 significantly, albeit not totally, blocked the effect of *Myd88* dsRNA on *Drosomycin-luciferase* reporter activity, indicating that endocytosis of dsRNA fragments is necessary for efficient transcriptional silencing in S2 cells.

We used immunofluorescence and electron microscopy to further characterize the uptake of FITC-dsRNA fragments into S2 cells (Fig. 3). dsRNA uptake is rapid; after 5 min, a major portion of the FITC-dsRNA was bound at the cell periphery just above the plasma membrane or internalized beneath the plasma membrane in small vesicles (Fig. 3).

**FIGURE 3.** dsRNA fragments are internalized efficiently by S2 cells. A. Fluorescent confocal microscopic images of internalization of FITC-dsRNA in S2 cells. FITC-dsRNA (500 bp) rapidly bound to the surface of S2 cells (5 min) and subsequently internalized into cytoplasmic vesicles (60 min). The nuclear DNA was stained using 4′,6-diamidino-2-phenylindole. B. Immunoelectron microscopic images of dsRNA localization in S2 cells. Exogenous FITC-dsRNA was detected employing a polyclonal antibody against FITC. 5 min after addition of FITC-dsRNA, the signal was seen predominantly on the cell surface and in small vesicles (size <1 μm) but not in larger vesicles. After 30 min, the signal almost entirely gathered to the walls of large intracellular vesicles (diameters, 1–2 μm).

Scavenger receptors are a group of structurally unrelated molecules known to mediate the endocytosis of certain polyanionic ligands, including nucleic acids (reviewed in Ref. 24). The *Drosophila* genome contains several scavenger receptors, some of which have been characterized in detail (20, 25–27). We tested whether RNAi targeting some of these receptors affected endocytosis of dsRNA (Fig. 4A). RNAi targeting the class B scavenger receptors *Croquemort* (*crq*), *emp*, and *ninA* as well as the *eater* homologues *CG8946* and *CG18146* had no effect on endocytosis, whereas RNAi targeting the class C scavenger receptor, *SR-CI*, and the epidermal growth factor repeat-containing scavenger...
receptor, *eater*, led to a significant decrease in the endocytosis of dsRNA fragments (500 bp) (Fig. 4A). Consistent with previous observations, *SR-CI* RNAi decreased also the endocytosis of AcLDL, whereas *eater* RNAi had a smaller effect (Fig. 4A and Refs. 26 and 27). A combination of *SR-CI* and *eater* RNAi resulted in >90% decrease in the internalization of dsRNA when compared with GFP dsRNA-treated controls. These data were confirmed by confocal microscopy (Fig. 4B); RNAi silencing of *SR-CI* and *eater* together led to a strongly reduced internalization signal. Targeting these receptors similarly decreased internalization of Alexa Fluor 488-labeled dsRNA, excluding the possibility that these scavenger receptors bind to fluorescein and not to dsRNA (data not shown). These results indicate that *eater* and *SR-CI* together account for a major part of the internalized dsRNA in S2 cells.

In contrast to *Drosophila* cells, mammalian cells do not readily internalize dsRNA fragments. Therefore, we tested whether expression of a *Drosophila* scavenger receptor can increase dsRNA internalization into mammalian cells. As shown in Fig. 5, CHO cells stably transfected with *SR-CI* (20) showed a marked increase in dsRNA uptake when compared with nontransfected control cells. Thus, expression of a *Drosophila* scavenger receptor is sufficient to confer increased uptake of dsRNA into mammalian cells.

**DISCUSSION**

We used an RNAi-based screen to find genes required for RNAi in *Drosophila* S2 cells. We identified a known RNAi component and three genes previously unknown to be involved in RNAi in *Drosophila*. The identification of Ago-2, the core component of the RISC (12), validates our screen and shows that it is suitable for identifying genes involved in RNAi. In addition, three other genes were identified: *belle*, *Pros45*, and *Chc*.

Interestingly, we noted that RNAi directed against *Ago-2*, *belle*, or *Pros45*, similar to control RNAi against *Dicer-2*, only partially rescued from the RNAi-induced lethal effect of *Ubi-p63E* knockdown (Fig. 1). Because it seems unlikely that all of these genes encode particularly long-lived proteins, this result suggests that none of these genes may be absolutely required for RNAi. Besides Belle, three putative RNA helicases (*Armitage, Spindle E, and Dmp68*) have been reported to be important for RNAi in S2 cells (28–30), and it is possible that one or all...
of these could compensate for reduced belle expression. Alternatively, it is conceivable that it may be technically impossible to entirely silence the expression of a gene encoding for a protein that itself is necessary for RNAi.

DEAD-box proteins are ATP-dependent RNA helicases that function at various stages of RNA processing (31). belle encodes a recently characterized Drosophila DEAD-box protein that is conserved from yeast to man (32). Strong mutations in Belle cause lethality by larval growth arrest and male and female sterility. Belle yeast ortholog DED1 is involved in translation initiation, ATP-dependent RNA unwinding, and remodeling of RNA-protein interactions (33–35). This makes Belle as well as Belle-related molecules an interesting candidate for unidentified siRNA duplex unwindase activities in Drosophila RISC.

Pros45, the Drosophila orthologue of SUG1, is one of the six ATPases in the base part of the regulatory subunit of the 26 S proteasome. The 19 S regulatory subunit confers polyubiquitin recognition to the 20 S proteasome core (36). A growing body of evidence indicates that besides its role in protein degradation, the 19 S regulatory proteasome subunit functions also in transcriptional regulation and in nucleotide excision repair and that the ATPase activity of SUG1 has been implicated in RNAi, and our RNAi machinery screen was ubiquitin-independent, we used an ubiquitin-independent RNAi assay to confirm that Pros45/SUG1 is required for efficient RNAi (Fig. 2A). Our results suggest that Pros45/SUG1 indeed may be involved in RNAi, either directly or indirectly, and warrant further investigation of its role in RNA silencing.

Our results indicate that endocytosis plays a role in the uptake of dsRNA in Drosophila cells. By a candidate approach, we were able to identify two endocytic receptors that together account for a major part of the dsRNA uptake in S2 cells, the previously characterized Drosophila scavenger receptors SR-CI and Eater. SR-CI and Eater are two structurally unrelated type I membrane proteins that have a role in the phago- cytosis of bacterial pathogens and display multi-ligand specificity reminiscent of mammalian scavenger receptors (26, 27). The binding activity of SR-CI has been mapped to its N terminus containing two complement control protein domains and one MAM (meprin/A5 anti- gen/mu receptor tyrosine phosphatase) domain. The ectodomain of Eater is predicted to contain 32 typical, non-calcium binding epidermal growth factor-like domains (27), the N-terminal four of which have been shown to participate in ligand binding. Our results suggest that dsRNA may be another, hitherto unrecognized, ligand for these molecules. It will be interesting to address whether the same domains bind all types of ligands. If so, further analysis of the structural basis of this interaction may help to provide insight into the basis of multiligand specificity in general.

Of note, the expression of both SR-CI and Eater has been shown to be restricted to plasmatocytes, a macrophage-like blood cell type of Drosophila (26, 27). For in vivo silencing approaches, which involve dsRNA injected into the hemolymph (blood equivalent of the fly) (40), one should therefore expect that a major part of the injected dsRNA fragments is cleared by plasmatocytes. This may have implications for this type of gene silencing approach in the fly.

The finding that mammalian cells show strongly increased internalization of dsRNA after expression of a Drosophila scavenger receptor (SR-CI) opens avenues for improved delivery of silencing agents into mammalian cells. Silencing agents such as siRNAs could be derivatized with scavenger receptor ligands, thereby facilitating their uptake and eliminating the need for transfections in cellular high throughput RNAi screens. It appears that SR-CI does not recognize siRNAs that are routinely used for RNAi in mammalian cells but rather longer fragments of dsRNA (supplemental figure and data not shown). Nevertheless, it is conceivable to explore direct delivery of 500 bp long dsRNA fragments into mammalian cells that carry deletions of RNase L and protein kinase R, a cell-based viral defense system for dsRNA that is thought to interfere with biological readouts (41, 42). This could facilitate mammalian high throughput RNAi screens especially with cell lines that are difficult to transfect by making such screens cheaper and more accessible.

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