Transport of dsRNA into Cells by the Transmembrane Protein SID-1

Evan H. Feinberg and Craig P. Hunter*

RNA interference (RNAi) spreads systemically in plants and nematodes to silence gene expression distant from the site of initiation. We previously identified a gene, *sid-1*, essential for systemic but not cell-autonomous RNAi in *Caenorhabditis elegans*. Here, we demonstrate that SID-1 is a multispan transmembrane protein that sensitizes *Drosophila* cells to soaking RNAi with a potency that is dependent on double-stranded RNA (dsRNA) length. Further analyses revealed that SID-1 enables passive cellular uptake of dsRNA. These data indicate that systemic RNAi in *C. elegans* involves SID-1–mediated intercellular transport of dsRNA.

RNAi in *C. elegans* initiated by injection of dsRNA spreads to silence the targeted gene throughout the animal and in its progeny (*1*). The mechanistic basis of this systemic transmission of gene silencing information is not understood. We previously identified *C. elegans* mutants deficient in systemic but not cell-autonomous RNAi (*2*). The first of these genes to be identified, *sid-1*, encodes a widely expressed putative transmembrane protein enriched at cellular membranes (*2*).

The predicted structure of SID-1 contains 11 transmembrane domains with a large (>400 amino acid) extracellular N-terminal domain and a ~100 amino acid loop residing between the first and second predicted transmembrane helices (*2*). Initially, we determined a portion of the membrane topology of SID-1. We generated *sid-1::lacZ* chimeric transgenes consisting of *sid-1* truncated after each of the predicted transmembrane domains and fused to *lacZ* or a synthetic transmembrane domain followed by *lacZ* (*3*–*5*) (Fig. 1). Fusion proteins in which β-galactosidase (β-Gal) is located in the cytosol should exhibit β-Gal activity, whereas fusion proteins in which β-Gal is located extracellularly should not exhibit β-Gal activity (*4*, *5*). Accordingly, we confirmed that the N-terminus of SID-1 is located extracellularly, that the C-terminus is located in the cytosol, and that five of the first six predicted transmembrane domains span the cell membrane (Fig. 1). A few truncations yielded inconclusive results, suggesting that truncations in certain regions destabilize SID-1 (*4*) (Fig. 1). The best studied loss-of-function allele of *sid-1*, *sid-1(qt2)*, encodes a single amino acid substitution at a residue within the fourth transmembrane domain, suggesting that the transmembrane domain sequences are essential to the function of SID-1.

Department of Molecular and Cellular Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA.

*To whom correspondence should be addressed. E-mail: hunter@mcb.harvard.edu

Previous analyses of *sid-1* mosaic animals indicated that *sid-1* is necessary for cell-autonomous import or processing of the RNAi signal in body wall muscle cells (*2*). More detailed molecular studies of SID-1 and the nature of the imported signal would be difficult in whole animals, therefore we expressed and studied SID-1 in a heterologous tissue culture system. *Drosophila* S2 cells were selected because *Drosophila* possesses robust cell-autonomous RNAi but lacks both a *sid-1* homolog and systemic RNAi (*2*, *6*, *7*). We generated expression plasmids containing C-terminally FLAG-tagged *sid-1* or the strong hypomorph *sid-1(qt2)*. Immunofluorescence of transfected S2 cells demonstrated that both proteins were expressed at similar levels and in indistinguishable patterns, apparently localized to both the cell membrane and cytosolic puncta, presumably corresponding to the endoplasmic reticulum and Golgi complex (fig. S1).

RNAi can be initiated in macrophage-like S2 cells by brief serum starvation in the presence of high concentrations of dsRNA, followed by incubation with serum for at least 72 hours (*8*, *9*). To distinguish this activity from SID-1–dependent activity, we omitted serum starvation, exposed cells to lower concentrations of dsRNA, and reduced the time between dsRNA addition and measurement of silencing. We cotransfected S2 cells with a plasmid encoding firefly luciferase and either *sid-1* or *sid-1(qt2)* plasmids, and we added 500 base pair (bp) luciferase dsRNA to their growth medium 48 hours after transfection. Luciferase activity was measured 24 hours after dsRNA addition. SID-1–expressing cells displayed a dsRNA dose-dependent silencing response and showed silencing comparable to cells expressing SID-1(qt2) at more than 10^5-fold lower dsRNA concentrations (Fig. 2A). Intriguingly, soaking in a luciferase small interfering RNA (siRNA), known to mediate complete RNAi when cotransfected into S2 cells, caused substantially less SID-1–dependent silencing (*10*) (Fig. 2D). To determine whether SID-1 is less active in transducing silencing communicated by siRNAs or shorter dsRNAs overall, we tested a series of dsRNAs of varying lengths spanning the canonical siRNA sequence. Silencing varied directly with the length of the dsRNA; longer dsRNA silenced much more potently than shorter RNA. For example, 500 bp dsRNA silenced as well as 100 bp dsRNA at approximately 100-fold to 1000-fold lower concentrations, and 100 bp dsRNA silenced as well as 21 bp siRNAs at approximately 10^5-fold lower concentrations (Fig. 2, A to D and F). A 100 bp control dsRNA corresponding to *C. elegans mex-3* had no effect on luciferase levels, indicating that the response observed was sequence-specific (Fig. 2E).

To determine the in vivo relevance of these findings, we conducted dsRNA sizing experiments in *C. elegans*. 100 bp dsRNA corresponding to *mex-3*, an essential embryonic gene expressed in the germ line, initiated germline RNAi when injected into either a single intestinal cell or directly into the germ line (*11*) (table S1). In contrast, 50 bp *mex-3* dsRNA effectively initiated RNAi only when injected directly into the germ line (table S1). To confirm the dependence on length rather than the presence of more effective siRNA sequences within the longer dsRNA, we constructed a chimeric 100 bp dsRNA that fused the 50 bp *mex-3* dsRNA sequence to 50 bp derived from

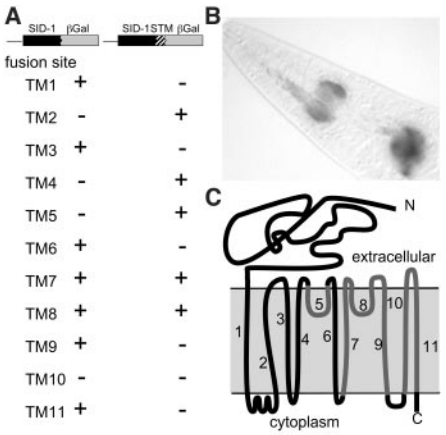

Fig. 1. SID-1 is a multispan transmembrane protein. (**A**) Schematic of the experimental design and result. β-Gal, with or without a leading synthetic transmembrane domain, was fused to the predicted nonmembrane region C-terminal to each predicted transmembrane domain (TM1, etc). (**B**) Constructs that place β-Gal in the cytoplasm produce active β-Gal. The *sid-1* promoter appears to be most active in the pharynx. The cumulative results from (A) indicate that TM domains 1 to 4 and 6 span the bilayer in the orientation shown (**C**). Predicted TM5 cannot span the bilayer and may be imbedded in the membrane, as shown, or may be extracellular. The topology and intracellular or extracellular location of the remaining predicted TM domains are not resolved by these results.

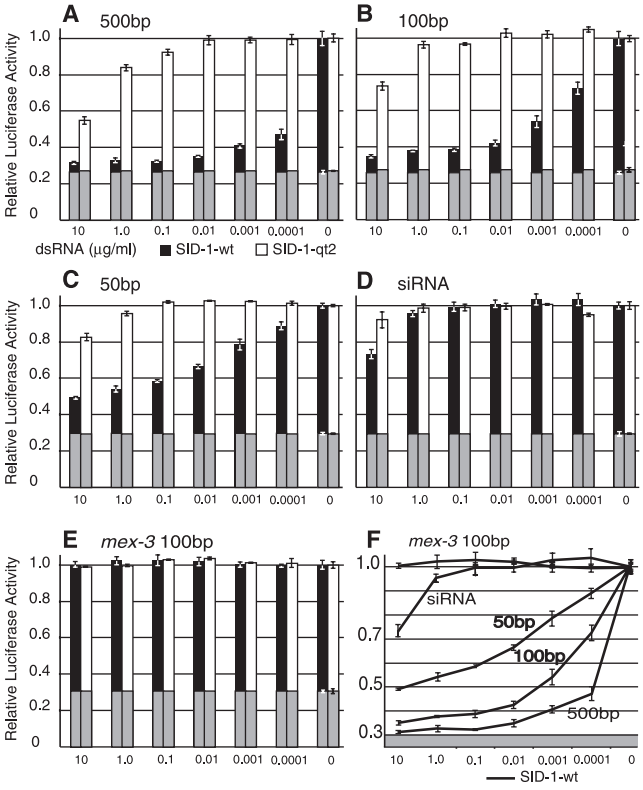

Fig. 2. SID-1 facilitates soaking RNAi in S2 cells. (**A** to **E**) Luciferase levels shown are relative to luciferase levels in cells exposed to no dsRNA. All treatments and measurements were made in triplicate, and error bars represent 1 SD. Gray bars inset within each column represent luciferase levels that remain at the end of the experiment in samples treated with the translation inhibitor cycloheximide (50 μg/ml) to control for protein stability. The standard deviation of the cycloheximide control is shown in the no-RNA bars only. (**F**) Summary of *sid-1* data from (A) to (E). Gray area indicates range of luciferase levels in cycloheximide-treated cells.

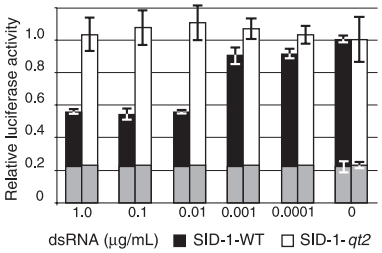

Fig. 3. SID-1 mediates dsRNA uptake. SID-1–mediated soaking RNAi in cl-8 cells as described in Fig. 2.

luciferase. This 100 bp dsRNA initiated germline RNAi when injected into either the germ line or the intestine (table S1). These data suggest that the dsRNA size discrimination observed in S2 cells is reflected in *C. elegans* and that longer dsRNAs function as preferred substrates for systemic RNAi.

The SID-1–dependent silencing observed in S2 cells is consistent with two potential mechanisms of SID-1 action: SID-1 may import dsRNA or it may simply increase the efficiency of RNAi independent of dsRNA uptake. To discriminate between these possibilities, we conducted similar luciferase silencing experiments in *Drosophila* cl-8 cells. Unlike S2 cells, cl-8 cells are refractory to soaking-induced RNAi but capable of potent RNAi when transfected with dsRNA (*12*). cl-8 cells expressing SID-1 displayed dose-dependent luciferase silencing in response to soaking in 500 bp luciferase dsRNA, whereas cl-8 cells expressing SID-1(qt2) were unresponsive to dsRNA even at the highest concentrations tested, thus serving as an internal control for the resistance of cl-8 cells to dsRNA soaking (Fig. 3). This result demonstrates that SID-1 enables soaking RNAi by facilitating dsRNA uptake and, importantly, that ectopically expressed SID-1 enables dsRNA uptake in multiple cell lines.

To address the mechanism of dsRNA uptake, we modified a commonly used approach to distinguish internalized ligand, in this case dsRNA, from surface-bound ligand. Cells were first incubated with ^{32}P-labeled dsRNA, then digested with trypsin to remove glycoproteins and any bound dsRNA from the cell surface, and finally measured for internalized radiolabel (*13*–*15*). Cells expressing SID-1 internalized 25- to 130-fold more dsRNA than cells expressing SID-1(qt2), confirming that SID-1 mediates its activity via import of dsRNA (Fig. 4).

We reasoned that dsRNA uptake could be mediated through four distinct mechanisms: (i) passive diffusion through a dsRNA channel or (ii) active transport via a pump, (iii) endocytosis, or (iv) phagocytosis. To determine whether SID-1 functions via a pump, endocytosis, or phagocytosis [three adenosine triphosphate (ATP)-dependent processes], we tested the effect of ATP depletion on dsRNA uptake (*16*). Cells expressing SID-1 or SID-1(qt2) were incubated with oligomycin to deplete ATP for 30 min before dsRNA was added. In ATP-depleted cells, nearly 300-fold more dsRNA was internalized by cells expressing SID-1 than by cells expressing SID-1(qt2), as compared with 30-fold more dsRNA internalized by mock-depleted cells expressing SID-1 than SID-1(qt2) (Fig. 4A). This indicates that SID-1–dependent uptake is substantially less sensitive to ATP depletion than the endogenous S2 uptake mechanism, presumably phagocytosis (Fig. 4A) (*8*). Similarly, in separate experiments conducted at 4°C, SID-1–expressing cells internalized approximately 2000-fold more dsRNA than cells expressing SID-1(qt2), whereas at 22°C SID-1–expressing cells internalized approximately 130-fold more dsRNA than SID-1(qt2)–expressing cells (Fig. 4B). These results have two important implications: First, the differing ATP and cold sensitivities of uptake in cells expressing SID-1 and SID-1(qt2) further bolster the argument that SID-1 imports dsRNA in a manner distinct from the endogenous uptake mechanism used by S2 cells. Second, SID-1 passively transports dsRNA into cells and, therefore, does not act as a pump or by endocytosis or phagocytosis. If SID-1 enables passive, diffusion-limited dsRNA uptake, then the accumulation of intracellular dsRNA should be rapid as the intracellular and extracellular dsRNA concentrations equilibrate. Indeed, SID-1–dependent uptake occurs rapidly, displaying distinct kinetics from SID-1–independent uptake (Fig. 4C).

In light of its sufficiency in *Drosophila* cells, its structure, and its passive nature of transport, SID-1 is likely to form a channel for dsRNA diffusion, although it is possible that SID-1 may enable dsRNA uptake indirectly by modifying the activity of other cellular proteins. Notably, both ATP depletion and cold incubation reduced recovered radiolabel by ~30 to 50%. We contend that this represents active retention of dsRNA that prevents diffusion of dsRNA out of cells during wash steps (Fig. 4, A and B). For example, internalized dsRNA could be retained by both ATP-dependent processes, e.g., after digestion by the ATP-dependent dsRNAse DICER and incorporation into ribonucleoprotein RISC complexes, and ATP-independent processes, such as binding to cellular dsRNA-binding proteins (dsRBPs) like RDE-4 (*17*, *18*). Thus, cellular energy depletion would affect the retention of a fraction of dsRNA.

Our data indicate that SID-1 enables transport of dsRNA in systemic RNAi. From the luciferase silencing results, we infer that SID-1 is more active in the transport of longer dsRNAs, although we observe a less potent but similarly graded response in cells expressing SID-1(qt2) or in cells transfected with luciferase alone (*19*) (Fig. 2, A to E). dsRNA length discrimination may be due to the tendency of RNA to weakly and nonspecifically bind a wide range of substrates, including cell surface gly-

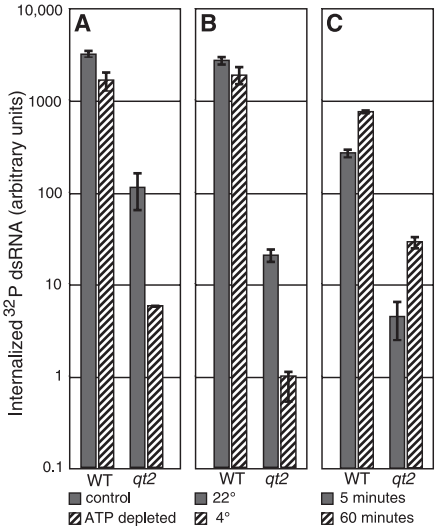

Fig. 4. SID-1 rapidly mediates passive uptake of dsRNA in S2 cells. (**A**) SID-1–mediated dsRNA uptake is resistant to ATP depletion. Cells (4×10^6) were plated in duplicate in 24-well plates in 5 μM oligomycin or dimethyl sulfoxide (DMSO) control for 30 min before addition of dsRNA. Cells were incubated with dsRNA for 1 hour at 22°C, washed once with cold phosphate-buffered saline (PBS), treated with trypsin for 15 min, pelleted, washed in PBS three times, and lysed. Radioactivity in lysates was measured and normalized to total protein content. Washing cells with acidified PBS was as effective as trypsin in removing extracellular label (*3*, *19*). (**B**) SID-1–mediated dsRNA uptake is resistant to reduced temperatures. Cells (4×10^6) were plated in duplicate, allowed to adhere for 30 min at 27°C, then moved to 4°C for 10 min. dsRNA was added, and cells either remained at 4°C or were warmed to 22°C. After 1 hour, both sets of cells were returned to 4°C, washed once with cold PBS, and trypsin was added. Both sets of cells were then moved to 22°C and processed as in (A). (**C**) SID-1–mediated uptake proceeds rapidly. Cells were plated as in (B) before addition of dsRNA and, after either 5 min or 60 min incubation (22°C), were washed and processed in (A).

coproteins. Low-affinity interactions with glycoproteins would be stabilized by increases in avidity that accompany lengthening of a dsRNA molecule. Longer molecules would bind more stably to the cell surface and thereby increase the concentration of dsRNA immediately surrounding the dsRNA channel. Such a discriminatory mechanism would enable organisms to use long dsRNA to systemically silence natural targets, including transposons or viruses, while spatially and temporally confining expression of short dsRNAs, such as regulatory microRNAs (*20*, *21*).

SID-1–mediated dsRNA transport may have numerous functional genomic and therapeutic applications. RNAi screens have been highly effective in *C. elegans* and S2 cells, which readily import dsRNA, whereas transfection-based RNAi screens require substantially more labor (*8*, *12*, *22*). Ectopic SID-1 expression may enable RNAi soaking screens in a number of experimental systems. Perhaps most important, *sid-1* belongs to a previously uncharacterized gene family with members within the human and murine genomes (*2*). Should these genes function similarly to *C. elegans sid-1*, modulation of their activity could enable in vivo use of RNAi to regulate gene expression.

References and Notes

1. A. Fire *et al.*, *Nature* **391**, 806 (1998).
2. W. M. Winston, C. Molodowitch, C. P. Hunter, *Science* **295**, 2456 (2002).
3. Materials and Methods are available as supporting online material at *Science* Online.
4. X. Li, I. Greenwald, *Neuron* **17**, 1015 (1996).
5. T. J. Silhavy, J. R. Beckwith, *Microbiol. Rev.* **49**, 398 (1985).
6. S. M. Hammond, E. Bernstein, D. Beach, G. J. Hannon, *Nature* **404**, 293 (2000).
7. N. Tavernarakis, S. L. Wang, M. Dorovkov, A. Ryazanov, M. Driscoll, *Nature Genet.* **24**, 180 (2000).
8. M. Ramet, P. Manfruelli, A. Pearson, B. Mathey-Prevot, R. A. Ezekowitz, *Nature* **416**, 644 (2002).
9. C. A. Worby, N. Simonson-Leff, J. E. Dixon, *Sci. STKE* **2001**, pl1 (2001).
10. S. M. Elbashir *et al.*, *Nature* **411**, 494 (2001).
11. B. W. Draper, C. C. Mello, B. Bowerman, J. Hardin, J. R. Priess, *Cell* **87**, 205 (1996).
12. L. Lum *et al.*, *Science* **299**, 2039 (2003).
13. D. S. Kwon, G. Gregorio, N. Bitton, W. A. Hendrickson, D. R. Littman, *Immunity* **16**, 135 (2002).
14. A. Shibuya *et al.*, *Nature Immunol.* **1**, 441 (2000).
15. K. Han *et al.*, *Mol. Cells* **12**, 267 (2001).
16. S. D. Conner, S. L. Schmid, *Nature* **422**, 37 (2003).
17. H. Tabara, E. Yigit, H. Siomi, C. C. Mello, *Cell* **109**, 861 (2002).
18. E. Bernstein, A. A. Caudy, S. M. Hammond, G. J. Hannon, *Nature* **409**, 363 (2001).
19. E. H. Feinberg, C. P. Hunter, unpublished data.
20. H. Tabara *et al.*, *Cell* **99**, 123 (1999).
21. V. Ambros, *Cell* **107**, 823 (2001).
22. R. S. Kamath *et al.*, *Nature* **421**, 231 (2003).

23. We wish to thank A. Kiger for pPacPl and advice concerning S2 cells; L. Lum and P. Beachy for cl-8 cells, growth supplements, and technical advice; M. Sutherlin and D. Mootz for critical reading of this manuscript; and P. Beachy and all members of the Hunter laboratory for helpful comments and suggestions. Supported by The Beckman Young Investigators Foundation and the National Science Foundation (MCB-01110452).

Supporting Online Material

www.sciencemag.org/cgi/content/full/301/5639/1545/DC1
Materials and Methods
Fig. S1
Tables S1 and S2
References

22 May 2003; accepted 31 July 2003

Orientation of Asymmetric Stem Cell Division by the APC Tumor Suppressor and Centrosome

Yukiko M. Yamashita,[1] D. Leanne Jones,[1] Margaret T. Fuller[1,2]*

Stem cell self-renewal can be specified by local signals from the surrounding microenvironment, or niche. However, the relation between the niche and the mechanisms that ensure the correct balance between stem cell self-renewal and differentiation is poorly understood. Here, we show that dividing *Drosophila* male germline stem cells use intracellular mechanisms involving centrosome function and cortically localized Adenomatous Polyposis Coli tumor suppressor protein to orient mitotic spindles perpendicular to the niche, ensuring a reliably asymmetric outcome in which one daughter cell remains in the niche and self-renews stem cell identity, whereas the other, displaced away, initiates differentiation.

Adult stem cells maintain populations of highly differentiated but short-lived cells such as skin, intestinal epithelium, or sperm through a critical balance between alternate fates: Daughter cells either maintain stem cell identity or initiate differentiation (*1*). In *Drosophila* testes, germline stem cells (GSCs) normally divide asymmetrically, giving rise to one stem cell and one gonialblast, which initiates differentiation starting with the spermatogonial transient amplifying divisions. The hub, a cluster of somatic cells at the testis apical tip, functions as a stem cell niche: Apical hub cells express the signaling ligand Unpaired (Upd), which activates the Janus kinase–signal transducers and activators of transcription (JAK-STAT) pathway within GSCs to maintain stem cell identity (*2*, *3*).

Analysis of dividing male GSCs by expression of green fluorescent protein (GFP)–α-tubulin in early germ cells revealed that in 100% of the dividing stem cells observed ($n > 500$), the mitotic spindle was oriented perpendicular to the hub-GSC interface throughout mitosis, with one spindle pole positioned within the crescent where the GSC contacted the hub (Fig. 1, A to C, and fig. S1). Stem cell division was rare, averaging one dividing stem cell observed per 5 to 10

[1]Department of Developmental Biology, [2]Department of Genetics, Stanford University School of Medicine, Stanford, CA 94305–5329, USA.

*To whom correspondence should be addressed. E-mail: fuller@cmgm.stanford.edu

Transport of dsRNA into Cells by the Transmembrane Protein SID-1

Evan H. Feinberg and Craig P. Hunter

Science **301** (5639), 1545-1547.
DOI: 10.1126/science.1087117