The dsRNA Binding Protein RDE-4 Interacts with RDE-1, DCR-1, and a DExH-Box Helicase to Direct RNAi in C. elegans

Hiroaki Tabara,1,3,5 Erbay Yigit,1 Haruhiko Siomi,2 and Craig C. Mello1,2,4
1Program in Molecular Medicine
2Howard Hughes Medical Institute
University of Massachusetts Medical School
Worcester, Massachusetts 01605
3Institute for Genome Research
University of Tokushima
Tokushima 770-8503
Japan

Summary

Double-stranded (ds) RNA induces potent gene silencing, termed RNA interference (RNAi). At an early step in RNAi, an RNaseIII-related enzyme, Dicer (DCR-1), processes long-trigger dsRNA into small interfering RNAs (siRNAs). DCR-1 is also required for processing endogenous regulatory RNAs called miRNAs, but how DCR-1 recognizes its endogenous and foreign substrates is not yet understood. Here we show that the C. elegans RNAi pathway gene, rde-4, encodes a dsRNA binding protein that interacts during RNAi with RNA identical to the trigger dsRNA. RDE-4 protein also interacts in vivo with DCR-1, RDE-1, and a conserved DExH-box helicase. Our findings suggest a model in which RDE-4 and RDE-1 function together to detect and retain foreign dsRNA and to present this dsRNA to DCR-1 for processing.

Introduction

The introduction of dsRNA can induce sequence-specific posttranscriptional silencing of cognate genes in a variety of organisms (reviewed in Cogoni and Macino, 2000; Vance and Vaucheret, 2001; Waterhouse et al., 2001). The experimental application of dsRNA to induce gene silencing has been termed RNA interference or RNAi (Fire et al., 1998). RNAi and related posttranscriptional gene silencing (PTGS) pathways have been implicated in silencing transposons (Tabara et al., 1999a; Ketting et al., 1999; Wu-Scharf et al., 2000) and/or viruses (Ratcliffe et al., 1997; Mourrain et al., 2000; Dalmay et al., 2001), suggesting that these pathways may function as a form of sequence-directed immunity.

PTGS pathways share features with a developmental gene regulatory pathway that involves natural dsRNA-encoding genes, recently named micro-RNA (miRNA) genes (Lau et al., 2001; Lee and Ambros, 2001; Lagos-Quintana et al., 2001). During PTGS, introduced dsRNAs, or dsRNAs synthesized from aberrant transgene transcripts, are processed into small RNAs of approximately 22–26 nucleotides that have been termed small interfering RNAs (siRNAs) because they are thought to guide the destruction of complementary target mRNAs (reviewed in Baulcombe, 2002). Natural miRNA genes encode RNA products of approximately 70 nucleotides which are predicted to fold into stable stem-loop structures that are processed into mature ~22 nucleotide miRNAs. The founding members of the miRNA gene family, lin-4 and let-7, encode miRNA products that appear to regulate translation during C. elegans development by base pairing with complementary sequences located in the 3’ UTRs of their target mRNAs (Lee et al., 1993; Wightman et al., 1993; Reinhart et al., 2000).

Genetic and molecular studies have uncovered similarities in the processing and function of miRNA and siRNA species. For example, both the RNAi pathway and miRNA pathway require related genes. In C. elegans, RNAi requires the gene rde-1, which encodes a member of a functionally novel but highly conserved eukaryotic gene family (Tabara et al., 1999a) whose members have also been implicated in gene silencing in fungi, plants, and Drosophila (Catalanotto et al., 2000; Fagard et al., 2000; Hammond et al., 2001). Homologs of rde-1 have also been implicated in development in Drosophila, C. elegans, and Arabidopsis (reviewed in Benfey, 1999). A recent study has shown that two C. elegans homologs of rde-1, named alg-1 and alg-2, are required for the processing and function of the lin-4 and let-7 miRNAs (Grishok et al., 2001). Similarly, studies in Drosophila, C. elegans, and in vertebrate cells have shown that the multifunctional RNaseIII-related enzyme Dicer is required for the proper processing of both the dsRNAs that trigger RNAi (Bernstein et al., 2001; Ketting et al., 2001) and the miRNA precursors whose products mediate developmental gene regulation (Grishok et al., 2001; Hultvag et al., 2001; Ketting et al., 2001).

The convergence between PTGS pathways and pathways that control developmental gene regulation raises the questions of what other features these pathways share and, importantly, what factors function uniquely within each pathway to guide RNA recognition and targeting. In the present study we show that rde-4 encodes a dsRNA binding protein required for RNAi. Interestingly, RDE-4 is not required for other PTGS pathways or for developmental pathways, suggesting that RDE-4 activity may function at a step unique to the RNAi pathway. We show that the stable interaction between RDE-4 and dsRNA requires rde-1(+ ) activity and that RDE-4 interacts with the RDE-1 protein in vivo. RDE-4 protein also interacts in vivo with DCR-1 and with a conserved DExH-box helicase that is required for RNAi in C. elegans. Interactions were not detected between RDE-4 and siRNAs, or miRNA precursors. RNA sequences bound to RDE-4 were restricted to regions found within the trigger dsRNA, and thus RDE-4 does not appear to interact with the mRNA or with dsRNA sequences amplified from the target mRNA. Our findings suggest that RDE-4 functions during the initial steps of RNAi to recognize foreign dsRNA and to present this dsRNA to DCR-1 for processing.
Results

Molecular Identification of rde-4

In previous genetic screens for RNAi-deficient mutants, we found that many C. elegans mutants deficient in RNAi also exhibit additional phenotypes including activation of transposons in the germline, a low penetrance sterile phenotype, and meiotic chromosome nondisjunction phenotypes (Tabara et al., 1999a). These findings suggested that RNAi may play a role in transposon silencing and in other essential functions in the germline. However, paradoxically, the two strongest mutants, rde-1 and rde-4, were completely deficient in RNAi but failed to exhibit transposon activation or any other discernible phenotypes. Subsequent genetic studies suggested that rde-1 and rde-4 may function upstream in the initiation of RNAi (Grishok et al., 2000).

To shed more light on the role of the RDE-4 protein in RNAi, we set out to clone the corresponding gene. We first mapped the gene to a small genetic interval and then assayed corresponding DNA sequences from the interval for rescue of rde-4 (see Experimental Procedures). rde-4 rescue was mapped to two overlapping RDE-4 clones, T20G5 and E03F9, and to the single corresponding open reading frame T20G5.11 (Figure 1A). We next sequenced the T20G5.11 gene in each of the rde-4 mutant strains and found point mutations predicted to disrupt the open reading frame of T20G5.11 in each allele. Two alleles, ne299 and ne301, have an identical lesion, a 1 base insertion creating a premature stop codon at the end of the first exon of T20G5.11 (Figure 1A). These two alleles were isolated from a single large clonal population during a screen for spontaneous mutants (Tabara et al., 1999a) and may thus reflect a single spontaneous lesion (we will henceforth refer to this allele as ne299). A second allele of rde-4, ne337, was identified in a screen using chemical mutagenesis and induces a premature stop codon at amino acid residue 229 of the T20G5.11 product (Figure 1A). The rde-4 gene is predicted to encode a 385 amino acid protein that contains two copies of a conserved motif found in dsRNA binding proteins (Figures 1B and 1C). The genetic lesions in rde-4 appear likely to be null mutations, and consistent with this idea the ne299 allele, when placed in trans to a chromosomal deficiency, exhibited an RNAi-deficient phenotype and no other additional phenotypes (data not shown).

RDE-4 Interacts with dsRNA In Vitro and In Vivo

The RDE-4 protein contains two copies of a dsRNA binding motif (dsRBM) that is found in numerous other proteins that interact with dsRNA in a sequence-non-specific manner (reviewed in Fierro-Monti and Mathews, 2000). Therefore, we first asked whether recombinant RDE-4 could bind to dsRNA by using an in vitro gel shift assay. Labeled dsRNA corresponding to a portion of a GFP cDNA was incubated with GST-fused RDE-4, or with the GST protein alone, and was then run on a native polyacrylamide gel. The GST-fused RDE-4 efficiently bound to the dsRNA, causing a mobility shift, while the GST protein did not (data not shown). In competition assays, dsRNA but not single-stranded RNA efficiently competed for RDE-4 binding (data not shown). Similarly, we asked whether the labeled dsRNA could bind to the
Figure 2. RDE-4 Binds dsRNA In Vivo

(A and B) Wild-type or rde mutant animals were exposed to pos-1 dsRNA by feeding on E. coli expressing pos-1 dsRNA for 15 min or 2 days (as indicated). The E. coli feeding strain used expressed a dsRNA, which corresponds to 85% of the pos-1 mRNA (as indicated in the diagram in (E)). After immunoprecipitation (IP) with anti-RDE-4 antibody (H11001) or with the IgG fraction obtained from the pre-immune serum (H11002), coprecipitated RNA was fractionated on 1.8% denaturing agarose gels (A) or on a 15% polyacrylamide sequencing gel (B). RNA species were blotted and analyzed by Northern hybridization with sense RNA probes (W, Y) and antisense RNA probes (X, Z) as illustrated in (E). The hybridizations in (A) and (B) were done with the same stringency. The probes Y and Z were prepared from a region not contained in the bacterial feeding vector. To remove single-stranded RNA, the RNA sample in lane 14 (asterisk) was treated with 10 μg/ml RNaseA and 0.5 μg/ml RNaseT1. The blot in (B) was hybridized with the sense RNA probe (W).

(C) Northern analysis of RDE-4::IPs prepared from wild-type animals and transgenic animals expressing an unc-22-specific dsRNA segment. The transgenic C. elegans line was designed to express a 1400 nt segment of unc-22, indicated by the black bar in the diagram (E), as both sense and antisense RNA. After immunoprecipitation with the anti-RDE-4 antibody, coprecipitated RNA was fractionated on a 1.2% denaturing agarose gel. Northern hybridization was performed with sense RNA probes U and T as illustrated in (E). The size of the unc-22 RNA associated with RDE-4 was approximately 2800 nt, suggesting that the transgenes express dsRNA with a hairpin structure.

(D) RDE-4 does not interact detectably with siRNA. Extracts prepared from wild-type animals either not exposed to dsRNA (lane 3) or exposed to pos-1 dsRNA by feeding for 2 days (lanes 4, 5, and 6) were subjected to immunoprecipitations as described above. RNA species were recovered from each fraction and were run on a 12% polyacrylamide sequencing gel. The RNA species were analyzed by Northern hybridization with a sense RNA probe (W). Hybridization was done with a moderate stringency to allow detection of 25 nt RNA species. As controls, lane 1 contained a synthetic RNA oligo corresponding to 21 nt of antisense pos-1 sequence, and lane 2 contained size-marker RNA.

(E) Diagrams representing the pos-1 and unc-22 mRNAs are shown as gray bars. The regions targeted with dsRNA are indicated by the solid black bars above each diagram. Probes used in the experiments are indicated by arrows beneath the diagrams.

recombinant RDE-4 immobilized on a membrane. This North-Western blot analysis also showed that the dsRNA binds efficiently to the GST-fused RDE-4 but not to the GST protein alone (data not shown).

We next wished to ask if RDE-4 interacts with dsRNA during RNAi in vivo. To address this question, polyclonal antibodies were raised against a recombinant RDE-4 protein. We then exposed animals to dsRNA targeting the gene pos-1 (Tabara et al., 1999b). We prepared extracts from these animals and from control animals not exposed to dsRNA, and we used RDE-4-specific antibodies to precipitate the RDE-4 protein complex. The precipitates were extracted to purify any associated RNA and analyzed by agarose and acrylamide gel electrophoresis followed by Northern blotting using pos-1 sense and antisense radiolabeled RNA probes. Both the sense and antisense probes detected approximately equal amounts of pos-1 RNA that migrated below 350 bases on an agarose gel (Figure 2A, lanes 3 and 11) and migrated in a size range of 50 to 200 bases on an acrylamide gel (Figure 2B, lane 1). The coprecipitated pos-1 RNA was resistant to treatment with a mixture of the single-strand-specific RNaseA and RNaseT1 enzymes (Figure 2A, lane 14), suggesting that the pos-1 RNA is double stranded. Little or no pos-1 RNA coimmunoprecipitated with RDE-4, which was purified from populations either not exposed to dsRNA or exposed for only 15 min (Figure 2D, lane 3; Figure 2A, lane 1). As expected, rde-4(ne299) mutants failed to exhibit pos-1 dsRNA in the precipitate (Figure 2A, lane 5). The RDE-4 immune complex recovered from the rde-1(ne300) and rde-3(ne298) mutant strains exhibited greatly reduced quantities of pos-1 dsRNA (Figure 2A, lanes 7 and 9).

Target mRNA and siRNA Sequences Do Not Coprecipitate with RDE-4
dsRNA is thought to exist and function at multiple steps in RNAi. First, it functions in the initiation step when long dsRNA is recognized as foreign. Second, it functions in the execution step when target mRNA is bound by siRNA...
from the region located just 5’ of the trigger sequence can be amplified (Lipardi et al., 2001; Sijsen et al., 2001). In order to ask if the RDE-4 immune complex contains antisense RNA sequences derived from regions of the mRNA located 5’ of the trigger dsRNA, we utilized transgenic animals that express dsRNA corresponding to a portion of the unc-22 gene (see Figure 2E). RNA probes were then used to assay for sequences in the RDE-4 immune complex that were derived either from the trigger region or from the region located just 5’ of the trigger. As expected, the RDE-4 immune complex contained unc-22 antisense RNA corresponding to the targeted region of unc-22 (Figure 2C, lane 5). In contrast, no significant hybridization was observed when a sequence derived from the region located just 5’ of the trigger dsRNA was used as a probe (Figure 2C, lane 1). As expected, precipitates prepared from nontransgenic wild-type animals did not exhibit significant hybridization to the probes designed to detect unc-22 antisense RNA (Figure 2C, lanes 3 and 7).

Finally, in order to ask if siRNA sequences precipitate with RDE-4, we fractionated the RNA species in the RDE-4 immune complex and in the RDE-4-depleted supernatant by acrylamide gel electrophoresis. We then analyzed RNA species smaller than 150 bases by Northern blotting using a pos-1 sense probe. We found that the RNA species in the supernatant included abundant molecules of less than 150 bases, including a prominent signal corresponding in size to siRNAs, that migrated at approximately 24 to 25 bases (Figure 2D, lane 6). RNA species of 24 to 25 bases were absent in the RDE-4 immune complex, suggesting that RDE-4 preferentially interacts with longer dsRNAs (Figure 2D, lane 5). Taken together, these studies indicate that RDE-4 binds to long dsRNA sequences that are restricted to the region present within the trigger dsRNA. These findings support a model in which RDE-4 interacts with the trigger dsRNA and functions in the initiation step of RNAi.

dsRNA Bound to RDE-4 Accumulates during RNAi

In order to determine what fraction of the total dsRNA was bound to RDE-4 and to examine the requirements for the stability of the dsRNA bound to RDE-4, we next analyzed RNA extracts from wild-type and rde mutant strains. Wild-type animals showed an accumulation of antisense and sense RNA corresponding to the pos-1 trigger dsRNA sequence that appeared to be very similar to the dsRNA sequences found in the RDE-4 immune complex (Figure 3A, lanes 3 and 12). This RNA species was similar in size but slightly smaller, on average, than the major pos-1 RNA species detected in the bacterial expression strain used to induce RNAi (Figure 3A, lane 9), suggesting that some digestion of the bacterially expressed dsRNA may have occurred during transit through the intestine of the animals. Single-stranded RNA-specific nucleases digested the endogenous pos-1 mRNA but failed to digest the accumulated RNA species (Figure 3A, lanes 19 and 20), suggesting that the accumulated RNA species is indeed double-stranded.

Interestingly, the analysis of total RNA extracts
showed that animals lacking rde-1, rde-3, and rde-4 activities exhibited markedly reduced levels of dsRNA. In contrast, animals lacking rde-2 and mut-7 accumulated dsRNA to levels that were intermediate (rde-2) or similar to wild-type (mut-7) (Figure 3A). The finding that the accumulation of this dsRNA species requires some but not all rde(+1) activities supports the idea that this RNA species is an intermediate in the RNAi process.

To ask what fraction of the accumulating dsRNA in the total RNA extract from wild-type animals is associated with RDE-4, we repeated the immunoprecipitations with RDE-4-specific antibodies or with control nonspecific IgG obtained from preimmune serum and compared the amount of the dsRNA present in the immune complex and supernatant of each lysate. We found that approximately 60% of the pos-1 dsRNA species present in the total RNA coprecipitates with the RDE-4 immune complex (Figure 3B, compare lanes 3 and 4), indicating that a majority of the accumulating dsRNA is associated with RDE-4.

RDE-4 Interacts with RDE-1 In Vivo
Our previous studies have suggested that RDE-1 and RDE-4 function at an upstream step in the initiation of RNAi (Grishok et al., 2000). This previous observation, and the finding that RDE-1 and RDE-4 are both required for the accumulation of dsRNA, prompted us to ask if RDE-4 forms a complex in vivo with RDE-1. For these studies we generated a transgenic strain expressing RDE-1 tagged with the HA epitope and RDE-4 tagged with the FLAG epitope. We first confirmed that these tagged proteins were functional and able to rescue the corresponding mutants (data not shown). We then immunoprecipitated RDE-4 with RDE-4-specific polyclonal antibodies, and RDE-1 via the epitope tag, and analyzed the precipitates by immunoblotting (Figure 4A).

In reciprocal assays, RDE-1 and RDE-4 were found to coprecipitate. Based on a comparison of the RDE-1 present in total extracts and in the RDE-4 immune complex, we estimate that approximately 5% of the RDE-1 molecules stably associate with the RDE-4 complex. The interaction between RDE-1 and RDE-4 occurred in animals that were not exposed to exogenous dsRNA, and the interaction was not abolished by treatment with either double-stranded RNA-directed nuclease, RNaseV1, or a mixture of RNaseA and T1 that are single-stranded RNA-specific nucleases (data not shown). These findings suggest that RDE-1 and RDE-4 form a complex in the absence of dsRNA, but cannot rule out the possibility that dsRNA promotes the interaction or is required for the interaction but is protected from digestion in the immune complex.

RDE-4 Forms a Complex with DCR-1 In Vivo
In order to identify additional proteins that interact with RDE-4 in vivo, we isolated large quantities of the RDE-4 immune complex and then resolved its components using SDS polyacrylamide gel electrophoresis (SDS-PAGE). For this experiment we used rde-4(ne299) mutant animals that were rescued via an RDE-4::GFP fusion protein. We then passed protein extracts prepared from these transgenic animals and from control wild-type animals over a column conjugated with anti-GFP monoclonal antibody. After electrophoresis, the gel was silver stained to detect components of the RDE-4::GFP immune complex (Figure 4C). These experiments identified prominent bands migrating at 210 kilodaltons (kDa) and 110 kDa as well as several additional polypeptides that were present in the RDE-4::GFP immune complex but absent in wild-type control animals. A minor band at 125 kDa was identified, corresponding in size to RDE-1, but contained insufficient protein for further analysis (Figure 4C). The 210 kDa and 110 kDa bands were subjected to trypic digestion, and the resulting fragments were then analyzed by mass spectrometry. The peptide mass fingerprints of p210 and p110 were compared with in silico-digested sequences from protein databases (see Experimental Procedures). A total of 21 peptides from the trypic digestion of p210 matched DCR-1 (Table
While 11 peptides from p110 matched a DExH-box RNA helicase-related protein F15B10.2 (Table 1, see below),

The finding that RDE-4 forms a complex in vivo with DCR-1 suggests that RDE-4 may function to present the foreign trigger dsRNA to DCR-1 for processing. DCR-1 also acts to process the stem-loop precursors of the developmental regulators lin-4 and let-7 (Grishok et al., 2001; Ketting et al., 2001). However, rde-4 mutants do not exhibit developmental defects (Tabara et al., 1999a), and thus are not required for the activities of lin-4 or let-7. Further, we did not observe lin-4 or let-7 precursor RNAs associated with the RDE-4 immune complex (data not shown). Thus, although RDE-4 interacts strongly with DCR-1 in vivo, RDE-4 is not essential for the developmental functions of DCR-1 and may rather serve as an adaptor protein that recruits DCR-1 to the RNAi pathway.

### A Conserved DExH-Box Helicase Required for RNAi

The RDE-4 protein interacts with a 110 kDa protein, which as described in the previous section corresponds to the predicted gene F15B10.2 (Table 1), one of two closely related C. elegans DExH-box RNA helicase genes. F15B10.2, along with its homolog C01B10.1, map directly adjacent to one another on chromosome IV and appear likely to be expressed together from a single promoter. Consistent with this idea, we found that some F15B10.2 cDNAs were spliced to the trans-spliced leader sequence (sl2), which is often spliced onto the 5′ end of transcripts encoded by downstream genes in an operon (reviewed in Blumenthal, 1995). We found that the C01B10.1 gene and a cDNA clone (yk226c6) both appear to encode a frame-shifted open reading frame predicted to terminate the protein prior to the helicase domain, raising the question of whether or not this represents a functional gene. An alternative conceptual splicing of C01B10.1, or utilization of an alternative initial methionine, could encode a protein with overall 74% identity to F15B10.2 (Figures 5A and 5B).

These C. elegans genes have close homologs in mammals, including a gene named RHIV-1 induced in response to viral infection in pigs (Figures 5A and 5B: Zhang et al., 2000). Notably, these helicase proteins are more similar to the DCR-1 helicase domain than to any other helicase-related proteins in C. elegans or other organisms (Figures 5A and 5B). Consequently, we have named these genes drh-1 and drh-2 (for F15B10.2 and C01B10.1, respectively) which stands for dicer-related helicase.

To ask if these genes might function in RNAi, we used RNAi to target their inactivation. Previous work on dcr-1 has shown that RNAi targeting dcr-1 causes developmental defects and can also partially inhibit RNAi (Grishok et al., 2001). Although the helicase domain of DCR-1 is similar to DRH-1 and DRH-2 at the protein level, the dcr-1 gene does not contain regions of extended nucleotide identity required for crossinference in an RNAi assay. The drh-1 and drh-2 genes, on the other hand, are more than 81% identical at the nucleotide level and contain several regions of 40 to 200 nucleotides with 100% identity, and are thus expected to crossinference in the RNAi assay. There are no extensive regions predicted to be unique to either helicase gene and thus it is doubtful that effective single RNAi is possible. We
The RDE-4 Protein and RNAi in C. elegans

Figure 5. DRH-1 and DRH-2 Are Helicase-Related Proteins Required for RNAi

(A and B) Alignment of DRH-1 (F15B10.2), DRH-2 (C01B10.1), D2005.5, RHIV-1, and DCR-1. (A) Alignment of DExH/D domains, and (B) alignment of the helicase conserved C-terminal domains. Identities shared with DRH-1 are shaded in black, and identities among the other proteins are shaded in gray. Asterisks above residues in (A) indicate the location of DExH/D motif.

(C) Reduced sensitivity to RNAi following drh-1 and drh-2(RNAi).

also targeted D2005.5, which, other than DCR-1, is the next most similar C. elegans helicase-related gene (Figures 5A and 5B).

We did not observe any developmental abnormalities in animals exposed to drh-1 or drh-2 dsRNA, injected either singly or in combination. However, we found that injection of either drh-1 or drh-2 (or both) dsRNAs rendered the injected animals resistant to RNAi targeting a second gene (Figure 5C). Both germline and somatic RNAi was dramatically reduced in the helicase RNAi animals. RNAi targeting D2005.5 did not induce developmental or RNAi defects (Figure 5C and data not shown). These results suggest that the DExH-box helicase DRH-1 (and perhaps DRH-2) not only interact with RDE-4 but are also required for RNAi in C. elegans.

Discussion

The RDE-4 Protein and the Initiation of RNAi

Recent work in numerous organisms has shown that RNAi and other forms of sequence-directed immunity share features both with each other and with natural developmental regulatory pathways. For example, both the foreign dsRNAs that trigger RNAi and the endogenous miRNA precursors that function in development are processed into small RNAs by a multifunctional RNaseIII-related enzyme, Dicer (DCR-1). These small RNAs (siRNAs and miRNAs) are thought to be incorporated into silencing complexes that mediate, respectively, mRNA destruction during RNAi or translational control during development. These findings raise two kinds of interesting question. First, what additional genes and mechanisms do these pathways share? And second, what factors function uniquely within each pathway to guide RNA recognition and targeting? In the present study we have described the dsRNA binding protein RDE-4, which appears to be dedicated to dsRNA recognition within the RNAi gene-silencing pathway. We have shown that RDE-4 forms a complex in vivo with DCR-1. However, the RDE-4 protein is not required for development and does not interact detectably with miRNAs in vivo. These findings suggest a model in which RDE-4 functions as a sensor that recognizes foreign dsRNA and brings this RNA to DCR-1 for processing (Figure 6A).

The RDE-4 protein contains two copies of the dsRNA binding motif (dsRBM) and no other recognizable functional domains. The crystal structure of a dsRBM bound to dsRNA reveals extensive contacts between this protein motif and the backbone of the double-helical RNA (Ryter and Schultz, 1998). Thus, dsRBMs are expected to interact nonspecifically with dsRNAs of any sequence. Several groups of dsRBM-containing proteins can be distinguished by the presence of additional conserved motifs (reviewed in Fierro-Monti and Mathews, 2000). Members of one such group, the dsRNA-activated protein kinases (PKRs) in mammals, contain a kinase motif and two dsRBMs. PKRs have previously been shown to be involved in the response to foreign dsRNA (reviewed in Proud, 1995). PKRs are activated by binding dsRNA, and they trigger signal transduction pathways that direct a general repression of translation and ultimately induce apoptosis. Thus, PKRs are thought to provide a first line of defense for clearing viral infection in mammals. Like PKRs, RDE-4 appears to play an upstream role in the recognition of foreign dsRNA. However, instead of a general translation arrest, RDE-4 initiates the sequence-specific destruction of complementary mRNAs.

The RDE-1 Protein and the Initiation of RNAi

We have shown that the RDE-4 protein interacts with RDE-1 in vivo. The RDE-1 protein has numerous homologs in C. elegans and in other organisms, and members
of the RDE-1 family have been implicated in gene silencing and developmental pathways in plants, fungi, and animals. RDE-1 homologs in *Drosophila* and *Neurospora* are found in complexes that contain siRNA molecules (Hammond et al., 2001; Catalanotto et al., 2002). Furthermore, RDE-1 homologs are required for miRNA function in *C. elegans* (Grishok et al., 2001) and have recently been found in a complex with miRNAs in vertebrate cells (Mourelatos et al., 2002). Thus, RDE-1-related proteins appear to function in a variety of homology-dependent mechanisms that involve pairing between small guide RNAs and mRNAs.

The interaction we detect between RDE-4 and RDE-1 could reflect a transfer of siRNA products from the RDE-4/DCR-1 complex to an RNA-induced silencing complex (RISC) that contains RDE-1 (Figure 6A). However, we have shown that RDE-1 activity is required for the accumulation of dsRNA bound to RDE-4. Thus, if RDE-1 does function downstream in the execution step of RNAi, it must also function upstream at a step required for the retention of dsRNA (Figure 6A). It is also possible that both the initiation of RNAi and the subsequent destruction of target mRNA occurs within a single complex and that completion of the targeting steps may be required for the continued retention and accumulation of the trigger dsRNA molecules engaged by RDE-4.

The activities of RDE-1 and RDE-4 appear to protect the foreign dsRNA from entering a nonspecific dsRNA destruction pathway (Figure 6A). This finding suggests that the initiation complex containing RDE-4 may collect dsRNA faster than DCR-1 or other downstream factors can process the dsRNA. Conceivably, RDE-1 and RDE-4 function as adaptor proteins that regulate the entry of dsRNA into the RNAi pathway so as not to saturate the cell’s essential dsRNA-processing machinery (Figure 6A).

A DExH-Box RNA Helicase Involved in RNAi
We found a 110 kDa RNA helicase-related protein, DRH-1, in a protein complex with RDE-4 in vivo. Our analysis, using peptide mass fingerprinting, specifically identified DRH-1, but we cannot rule out the possibility that a small portion of the 110 kDa band contains a second very similar predicted protein named DRH-2. While numerous proteins related to DRH-1 are present in eukaryotes, DRH-1 is most similar to an unstudied human protein and to a porcine protein, RHIV-1. Interestingly, the *rhiv-1* gene was identified as a locus whose expression is activated in pigs infected with porcine reproductive and respiratory syndrome virus (PPRSV) (Zhang et al., 2000). PPRSV is a positive-strand RNA virus that forms dsRNA structures during its life cycle. The finding that DRH-1 is most similar to a family of vertebrate helicase proteins that include a protein activated in response to viral infection suggests that this family of proteins may have ancestral functions in the response to foreign dsRNA.

Up to the present, studies in other organisms have revealed several RNA helicases required for PTGS pathways. Mut6 in *Chlamydomonas* is a DExH-box RNA helicase involved in PTGS and is most similar to the *C. elegans* MOG-1 helicase required for sex determination in the germline (Wu-Scharf et al., 2000). SDE3 in *Arabidopsis* is required for PTGS and is similar to Upf1-type RNA helicases (Dalmay et al., 2001). A biochemical study of RNAi in *Drosophila* suggests that RNA helicase activity may be required downstream of siRNA formation (Nykanen et al., 2001). And another recent study in *C. elegans* has identified MUT-14 as a DExH-box helicase required downstream of siRNA formation for target mRNA destruction during RNAi (Tijsterman et al., 2002). The Dicer protein also contains an RNA helicase domain, which notably is most similar to the helicase domain of DRH-1.

We have found that RNAi targeting *drh-1* results in inhibition of RNAi in both the soma and germline but causes no other phenotypes. In contrast, mutations in *dcr-1* or RNAi targeting *dcr-1* inhibit RNAi but also result in lethal phenotypes (Grishok et al., 2001; Knight and Bass, 2001; Ketting et al., 2001). Thus, DRH-1 does not appear to be required for developmental functions of DCR-1 and may instead function closely with RDE-4 in a step specifically required for RNAi (Figure 6A). There are several possibilities for the role of DRH-1. For example, the helicase activity of DRH-1 may change the conformation of the dsRNA in order to facilitate the translocation of the protein complex along the dsRNA. Or it may facilitate transfer of the dsRNA from RDE-4 to the active site of DCR-1. It is conceivable that this additional helicase activity is necessary in order to pro-
cess the long-contiguous helices of the foreign dsRNAs that initiate RNAi. This activity may be less critical in processing miRNA precursors in which only short-contiguous helices are present (Lau et al., 2001; Lee and Ambros, 2001; Lagos-Quintana et al., 2001).

Distinct Initiation Steps in Related Gene Silencing Pathways

In C. elegans, the RNAi pathway overlaps genetically with the transposon-silencing and cosuppression pathways (Tabara et al., 1999a; Ketting et al., 1999; Plasterk and Ketting, 2000). Cosuppression is a PTGS silencing phenomenon that occurs when extra copies of a gene are introduced into an organism, resulting in the simultaneous silencing of both the transgene and homologous endogenous genes (reviewed in Cogoni and Macino, 1999). Transposon silencing may represent one in vivo function of the cosuppression pathway, as no genetic or experimental data provides evidence for a distinction between these pathways.

A previous study on the genetics of RNAi pathway genes suggested that rde-1 and rde-4 function upstream in a step that is unique to the RNAi pathway, while rde-2 and mut-7 function together at a downstream step common to the RNAi, transposon silencing, and cosuppression pathways (Grishok et al., 2000). Consistent with these findings, we have shown that during the initiation of RNAi, foreign dsRNA is recognized by RDE-4 and accumulates in tissues in a process that requires both RDE-4 and RDE-1. Furthermore, we have shown that RDE-2 and MUT-7, which appear to function in multiple silencing pathways, are not required for the accumulation of dsRNA during RNAi, consistent with a down-stream function for these gene products (Figure 6).

The finding that RDE-4 is a dsRNA binding protein that interacts with foreign dsRNAs raises the question of why this protein does not also recognize and initiate silencing in response to dsRNAs that are postulated to function in the transposon silencing and cosuppression pathways (reviewed in Plasterk and Ketting, 2000; Vance and Vaucheret, 2001; Waterhouse et al., 2001). One attractive possibility is that transposon silencing and co-suppression are triggered when transcription occurs within partially repressed chromatin (Figure 6B). Conceivably, abnormal transcripts produced from the repressed chromatin are converted into dsRNAs through the activity of an RNA polymerase dedicated to this transcription unit and thus binds the nascent mRNA of why this protein does not also recognize and initiate silencing in response to dsRNAs that are postulated to function in the transposon silencing and cosuppression pathways discussed in Timmons and Fire (1998) and Tabara et al. (1999a). RDE-4 is not likely to function in the recognition of the amplified dsRNAs. Perhaps amplification in the RNAi pathway involves the same hypothetical RNA polymerase complex postulated to function in the production of dsRNA transcripts from partially repressed chromatin (Figure 6). A good candidate for such a polymerase has been described as a factor required for cosuppression in both fungi and plants (Cogoni and Macino, 1999; Mourrain et al., 2000; Dalmay et al., 2000). However, surprisingly, members of this protein family are also required for RNAi in C. elegans (Smardon et al., 2001; Sijen et al., 2001; D. Conte and C.C.M., unpublished). These findings could indicate that amplification is required for RNAi even when abundant dsRNA is present. Alternatively, it is possible that members of this protein family do not function in dsRNA synthesis, or function at multiple steps, both in producing dsRNAs that trigger cosuppression and in later steps that occur downstream of the convergence between these silencing pathways.

Our study sheds light on the upstream events that distinguish RNAi from related gene-silencing pathways. A future challenge will be to elucidate the events unique to the initial steps in the cosuppression and microRNA pathways. These studies will no doubt lead to new insights into the world of small regulatory RNAs and should in turn lead to improved methods for controlling gene expression.

Experimental Procedures

Strains, Genetic Analysis, and Mapping

The Bristol strain N2 was used as a standard wild-type C. elegans strain. Genetic mapping placed rde-4(ne299) mutation to the right end of the interval between sqv-3(n2842) and unc-69(e587) mutations on chromosome III. Rescue experiments were performed by coinjection of the rescue construct with a mixture of plasmids that express sense and antisense unc-22 RNA as well as rol-6 transformation marker pRF4 as described in Tabara et al. (1999a). rde-1(ne300), rde-2(ne221), rde-3(ne298), rde-4(ne299), and mut-7(pk204) mutants were used to analyze the accumulation of dsRNA species during feeding RNAi. rde-4(ne337) was isolated by this work and utilized for the detection of a rde-4 mutation.

RNA Interference Assays

RNAi by microinjection was performed as described in Fire et al. (1998) and Rocheleau et al. (1997). RNAi by feeding was performed as described in Timmons and Fire (1998) and Tabara et al. (1999a).

Cosmid and cDNA Clones

Cosmid clones around rde-4 locus were obtained from A. Coulson. rde-4 cDNA clone yk333g4, drh-1 cDNA clone yk47b12, and drh-2 cDNA clone yk226c6 were obtained from Y. Kohara and used in this work.

Transgenic Worms Used for Immunoprecipitation

rde-1 and rde-4 genes were tagged with epitope sequences and cloned into plasmids. Instead of 145 lysine in RDE-1, two copies of transfor-those that interact with foreign dsRNAs raises the question of why this protein does not also recognize and initiate silencing in response to dsRNAs that are postulated to function in the transposon silencing and cosuppression pathways (reviewed in Plasterk and Ketting, 2000; Vance and Vaucheret, 2001; Waterhouse et al., 2001). One attractive possibility is that transposon silencing and co-silencing are triggered when transcription occurs within partially repressed chromatin (Figure 6B). Conceivably, abnormal transcripts produced from the repressed chromatin are converted into dsRNAs through the activity of an RNA polymerase dedicated to this transgene directly within the nuclei of target tissues (Tabara et al., 1999a). RDE-1 and RDE-4 are required for the initiation of RNAi in animals exposed to dsRNA, but not for interference inherited by progeny (Grishok et al., 2000). Thus, if an amplification mechanism functions downstream in the RNAi pathway (Lipardi et al., 2001; Sijen et al., 2001),
Antibody Production and Immunoprecipitation
A carboxy-terminal region (amino acid residues 218–386) of RDE-4 was tagged with 6× His and expressed in bacteria. Polyclonal rabbit antibodies were raised against the purified recombinant protein. The antibody was affinity purified using the recombinant protein immobilized to metal-chelated resin.

Before immunoprecipitation, animals were separated from the bacteria with sucrose flotation method and agitation in m9 buffer for 30 min to digest the bacteria remaining in guts. The animals were homogenized in lysis buffer (25 mM HEPES-NaOH [pH 7.4], 150 mM NaCl, 0.2 mM DTT, 10% glycerol, 1% Triton X-100 and complete protease inhibitors [Roche]). For RNA coprecipitation experiments, 2% Supersasein (Ambion) was included in the lysis buffer. The affinity-purified anti-RDE-4 antibody, anti-HA antibody 3F10 (Roche), and anti-FLAG antibody M2 (Sigma) were used for immunoprecipitation and immunoblotting. To recover RNA from RDE-4 immunoprecipitates, the precipitates were incubated in 0.2 mg/ml Proteinase K, 0.7× TBS, 5 mM EDTA, and 0.1% SDS for 30 min at 50°C and were extracted with phenol and chloroform. The recovered nucleic acids were analyzed by Northern hybridization essentially as described below.

Protein Identification by Peptide Mass Fingerprinting
To identify proteins interacting with RDE-4, the transgenic animals expressing RDE-4::GFP fusion protein were homogenized in the lysis buffer as described above. Anti-GFP monoclonal antibody 3E6 (Q-Biogene) was crosslinked onto Protein G-Sepharose beads with dimethyl pimelimidate. RDE-4::GFP immune complex was precipitated with the anti-GFP beads and was analyzed with SDS-PAGE.

The gels were visualized by silver staining or Coomassie staining. After Coomassie staining, 210 kDa and 110 kDa bands were excised from the gel and digested in gel with trypsin. The masses of the tryptic peptides were measured with matrix-assisted laser desorption ionization-reflector time-of-flight (MALDI-TOF) mass spectrometry (Shimazu Biotech, Inc., Japan). Searching of the mass values against the database was performed with the program MS-Fit at http://prospector.ucsf.edu (Clauser et al., 1999).

Northern Blot Analysis
For analyses of total RNA, animals exposed to pos-1 dsRNA by feeding were purified from the plates and homogenized in guanidine solution (4 M guanidine thiocyanate, 50 mM Tris [ pH 7.4], 10 mM EDTA, 0.65% laurylsarcosine, and 1% 2-mercaptoethanol). Nucleic acids were extracted from the homogenates with phenol and chloroform and were precipitated with sodium acetate and isopropanol. The precipitates were resuspended in water and were subsequently acidified with sodium acetate (330 mM [pH 4]). DNA was removed from the nucleic acids by extraction with phenol and chloroform under this acidic condition.

RNA samples were diluted with formamide/EDTA gel-loading buffer and were heat denatured before loading. Approximately 20 μg of total RNA was run per lane on a 1% agarose/formaldehyde gels. RNA species were transferred onto plus-charged nylon membranes from the gels with capillary blotting. Strand-specific riboprobes were synthesized using T7 or T3 RNA polymerase and [α-32P]UTP (380 Ci/mmol). Hybridization was performed at 57°C to 60°C in a hybridization buffer containing formamide. Subsequently, blots were hybridized with riboprobe in 50% formamide, 3× SSC, 1% SDS, 5% dextran sulfate, and 150 μg/ml yeast RNA. The blots were washed basically with 0.1× SSC/0.1% SDS at 65°C.

To detect low-molecular-weight RNA, RNA species were run on polyacrylamide/urea gels and transferred to membranes via semidry electroblotting. For riboprobes used for the detection of 25 nt RNA species, the radiolabeled probe was partially fragmented by boiling ble-stranded RNA in electrophoresis with 10% acrylamide/urea gels and transferred to membranes via semidry electroblotting. For riboprobes used for the detection of 25 nt RNA species, the radiolabeled probe was partially fragmented by boiling ble-stranded RNA in electrophoresis with 10% acrylamide/urea gels and transferred to membranes via semidry electroblotting.

References


Accession Number

The GenBank accession numbers are as follows: rde–4, AY071926; drh–1, AF480439; and drh–2, AF480440.