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Functional analysis of *dicer-2* missense mutations in the siRNA pathway of *Drosophila*

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Abstract

The *Drosophila* RNase III enzyme Dicer-2 processes double-stranded RNA (dsRNA) precursors into small interfering RNAs (siRNAs). It also interacts with the siRNA product and R2D2 protein to facilitate the assembly of an RNA-induced silencing complex (RISC) that mediates RNA interference. Here, we characterized six independent missense mutations in the *dicer-2* gene. Four mutations (P8S, L188F, R269W, and P365L) in the DExH helicase domain reduced dsRNA processing activity. Two mutations were located within an RNase III domain. P1496L caused a loss of dsRNA processing activity comparable to a null *dicer-2* mutation. A1453T strongly reduced both dsRNA processing and RISC activity, and decreased the levels of Dicer-2 and R2D2 proteins, suggesting that this mutation destabilizes Dicer-2. We also found that the carboxyl-terminal region of R2D2 is essential for Dicer-2 binding. These results provide further insight into the structure–function relationship of Dicer, which plays a critical role in the siRNA pathway.

Keywords

RNA interference; siRNA; *Drosophila*; Mutation; Dicer-2; R2D2

RNA interference (RNAi) is a biological process in which double-stranded RNA (dsRNA) triggers gene silencing in a sequence-specific manner [1]. RNAi can be divided into two phases. During the initiation phase, dsRNA is processed by a multidomain RNase III enzyme called Dicer, into 21–23 nucleotide (nt) siRNA duplexes. In *Drosophila*, Dicer-2 (Dcr-2) is responsible for generating siRNAs [2,3] and is present as a complex with the dsRNA-binding protein R2D2, which does not regulate the dsRNA processing activity of Dcr-2 [4].

During the effector phase, the siRNA product assembles into an RNA-induced silencing complex (RISC) via an ordered pathway by which one of the two RNA strands is selectively retained to direct RISC-mediated mRNA cleavage and degradation [5]. In *Drosophila*, RISC

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assembly is initiated by the formation of an R2D2/Dcr-2 initiator (RDI) complex, which corresponds to an R2D2/Dcr-2 heterodimer bound to a duplex siRNA [6,7]. The function of Dcr-2 in loading siRNA onto RISC is distinct from its role in siRNA production. Therefore, Dcr-2 plays crucial roles in both the initiation and effector phases of the siRNA pathway in *Drosophila*.

In the present study, we report the identification and characterization of six new *dcr-2* missense alleles that suppress constitutive RNAi against the *white* gene in *Drosophila*. Collectively, our results provide further insight into the structure–function relationship of Dicer.

Materials and methods

Drosophila genetics

Ethylmethanesulfonate (EMS)-generated *dcr-2* mutant lines were isolated from a genetic screen for genes involved in the RNAi pathway in *Drosophila* as previously described [2]. Mosaic analysis of the adult compound eye was performed as previously described [2]. To remove lethal background mutations, *dcr-2* mutants were recombined with the wild type Canton S strain and then maintained as homozygous stocks in a *GMR-wIR* background [2].

Sequencing

Genomic DNA was extracted from wild type and homozygous *dcr-2* mutant flies. All of the exons and flanking intronic regions of *dcr-2* were amplified by PCR, and the products were analyzed by DNA sequencing to identify sequence changes.

In vitro assays for RNAi

Drosophila embryo lysates were prepared by collecting 0–2 h embryos from wild type and homozygous *dcr-2* mutants as previously described [8]. Reagents and protocols used for dsRNA processing, target RNA cleavage, and RISC assembly assays were previously described [6].

Real-time RT-PCR

Total RNA was isolated from wild type and homozygous *dcr-2^{A1453T}* embryos using the TRIzol reagent (Invitrogen). Three micrograms of total RNA from each sample was treated with RNase-free DNase I (Invitrogen) to generate first-strand cDNA using a random hexamer (GE Healthcare) and Superscript III (Invitrogen) according to the manufacturer's protocol. The resulting cDNA samples were then used as templates for quantitative PCR using an iCycler iQ instrument (Bio-Rad). PCR was performed with iQ SYBR Green SuperMix (Bio-Rad) and the following gene-specific primers: *dcr-2*: 5'-CAGCGATTCTGATGAGTCT-3' (forward) and 5'-GTTGAGCAGCAAGCCATAGA-3' (reverse); *r2d2*: 5'-TTGAGGTAGTGCAGCAAAGC-3' (forward) and 5'-TTTTTGTCCGACTTTCCGTA-3' (reverse); and *RP49*: 5'-CCAAGCACTTCATCCGCCACCA-3' (forward) and 5'-GCGGGTGCGCTTGTTTCGATCC-3' (reverse). The quality of quantitative RT-PCR products was assessed by melting-curve analysis. All assays were done in triplicate and normalized to *RP49* RNA levels that served as an internal control.

Plasmid construction

To express either Dcr-2 or EGFP with an amino-terminal triple-HA epitope in *Drosophila* Schneider 2 (S2) cells, either an epitope-tagged *dcr-2* genomic DNA fragment or EGFP gene was inserted into pMK33. To express R2D2 or its deletion derivatives with an amino-

terminal triple-FLAG epitope, wild type *r2d2* cDNA and PCR products for the deletion mutants were inserted into the XbaI and BamHI sites of pBSKS-FLAG, and the NotI–BamHI fragment was then subcloned into pMK33 for expression in S2 cells. The nucleotide sequences of all constructs were confirmed by DNA sequencing.

Cell culture and transfection

S2 cells were cultured in HyQ SFX-Insect media (Hyclone) containing antibiotics. For transfection, 1-ml aliquots of the S2 cell suspension (5.0×10^5 cells/ml) were placed into each well of a 12-well tissue culture plate one day before transfection. The expression plasmid for HA-Dcr-2 or HA-EGFP was transiently co-transfected with the expression plasmid for FLAG-tagged, full-length R2D2 or its deletion derivatives into cells using Cellfectin (Invitrogen) according to the manufacturer's instructions. Expression was induced by the addition of CuSO_4 to a final concentration of 0.7 mM.

Immunoprecipitation (IP)

After expression was induced for 48 h, S2 cells were lysed in ice-cold cell lysis buffer [10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 50 mM NaF, 200 mM Na_3VO_4 , and complete protease inhibitor (Roche)] on ice for 30 min and centrifuged at 14,000g for 15 min at 4 °C. For IP, 200 μg of the clarified cell lysate was incubated with 20 μl anti-HA antibody conjugated to agarose beads (anti-HA affinity matrix, Roche) with rotation for 4 h at 4 °C. The beads were washed three times with IP wash buffer (30 mM Hepes–KOH, pH 7.2, 100 mM potassium acetate, 2 mM magnesium acetate, 5 mM DTT, and complete protease inhibitor). The bound fraction was eluted by boiling in 30 μl of 2 $\times$ SDS-sample buffer and used for Western blot analysis.

Western blotting

Western blot analysis was performed as previously described [6]. Anti-Dcr-2 and anti-R2D2 antibodies were provided by Q. Liu. Anti-HA and anti-FLAG antibodies were purchased from Roche and Sigma, respectively. Anti- β -tubulin antibody was obtained from the Developmental Studies Hybridoma Bank and was used to verify equal loading of protein on the gel.

Results and discussion

Identification of new *dcr-2* missense alleles

We previously isolated a viable complementation group consisting of 39 *dicer-2* (*dcr-2*) alleles from an EMS-induced mutagenesis screen of chromosome 2R for mutants in RNAi [2]. When adult compound eyes were made homozygous for the mutation [9,10], they showed defects in eye-specific RNAi induced by the *GMR-wIR* transgene against the endogenous *white* gene, as indicated by stronger eye pigmentation relative to a *GMR-wIR* fly (Fig. 1A). Dcr-2 contains an amino-terminal DExH-box ATP-dependent RNA helicase domain, a DUF283 domain, two neighboring RNase III domains (RNase IIIa and RNase IIIb), and a carboxyl-terminal dsRNA-binding domain, but lacks a PAZ domain (Fig. 1B). Among the 39 *dcr-2* alleles, we previously characterized six independent alleles including two null mutations [2]. Flies homozygous for the null *dcr-2* alleles were observed to be both viable and fertile [2]. However, the remaining 33 *dcr-2* mutants were homozygous lethal, indicating the presence of a second-site lethal mutation on the chromosome. Following the removal of lethal background mutations from these mutants by recombination, we obtained 9 *dcr-2* homozygous mutant flies. We sequenced the entire *dcr-2* open reading frame from each homozygote to identify new *dcr-2* missense alleles. Six of these alleles contained independent missense mutations that altered a single amino acid in Dcr-2. Four of these

missense mutations (P8S, L188F, R269W, and P365L) were located in the helicase domain, and two (A1453T and P1496L) were within the RNase IIIb domain (Fig. 1B). Amino acid residues P8, L188, A1453, and P1496 are highly conserved among Dicer orthologs, whereas the other residues (R269 and P365) are not (Fig. 1C; Supplementary Fig. S1). The eye-color phenotype of all six *dcr-2* missense alleles was not as strong as the *dcr-2* null phenotype, indicating that they are not null mutations (Fig. 1A).

***dcr-2* missense alleles are defective in siRNA production**

To determine whether the phenotype associated with the *dcr-2* missense alleles resulted from a defect in siRNA production, we performed an *in vitro* dsRNA processing assay. Embryo lysates from *dcr-2* mutants bearing missense mutations in the helicase domain showed slight reductions in dsRNA processing activity compared with wild type (Fig. 2A), which might reflect the *in vivo* phenotype of each mutant. Based on our previous observation that Dcr-2 requires a functional helicase domain for dsRNA processing [2], this result suggests that these missense mutations do not significantly impair the helicase activity of Dcr-2. Importantly, embryo lysates from the *dcr-2^{A1453T}* and *dcr-2^{P1496L}* mutants with the amino acid substitutions in the RNase IIIb domain were greatly impaired for dsRNA processing. The *dcr-2^{A1453T}* lysate retained approximately 15% of the wild type dsRNA processing activity, whereas the *dcr-2^{P1496L}* lysate was as impaired in siRNA production as the null *dcr-2^{L81IfsX}* lysate (Fig. 2A). Western blot analysis showed normal levels of Dcr-2 protein present in the *dcr-2^{P1496L}* mutant, whereas there was a large reduction in Dcr-2 protein levels in the *dcr-2^{A1453T}* mutant (Fig. 2B). The latter result might explain why the *dcr-2^{A1453T}* mutant had greatly reduced dsRNA processing activity. However, normal Dcr-2 levels in the *dcr-2^{P1496L}* mutant suggest the importance of P1496 in Dcr-2 RNase III activity. The crystal structures of the *Aquifex aeolicus* (Aa) RNase III-product complex and the protozoan *Giardia* Dicer [11,12], along with the biochemical analysis of recombinant human Dicer [13], have provided a single processing-center model for Dicer. In this model, two RNase III domains of Dicer form an intramolecular pseudodimer that resembles the homodimer of bacterial RNase III enzymes [14], and create a catalytic valley in which dsRNA substrates are cleaved by two catalytic sites on the opposite strands to generate products with 2-nt 3' overhangs. Magnesium ions are required for the cleavage reaction and are coordinated by invariant acidic residues. Additionally, the recent crystal structure of the single RNase IIIb domain of human Dicer showed that the domains could self-associate to form a stable homodimer, mimicking the intramolecular dimerization between the two RNase III domains of Dicer [15]. P1496 affected in the *dcr-2^{P1496L}* mutant is equivalent to the E64 residue of Aa-RNase III that is involved in substrate recognition and scissile-bond selection within the RNA-binding motif RBM 3 located on both ends of the catalytic valley [11], which is conserved in other RNase III domains (Fig. 1C). The homologous E673 residue in *Giardia* Dicer appears to be involved in substrate binding by coordinating a third metal ion at one end of the catalytic valley [12,15]. However, a non-acidic Pro residue at the equivalent position of Aa-RNase III E64 and *Giardia* Dicer E673 is mostly present in the RNase IIIb domains of Dicer orthologs (Fig. 1C). Interestingly, the Pro residue of human Dicer could be substituted by Glu without affecting its RNase III activity [13,15]. We suspect that the change in *Drosophila* Dcr-2 P1496 to an amino acid (Leu) with a side-chain property different from those of both Glu and Pro is not tolerated, and may interfere with dsRNA substrate positioning and/or scissile-bond selection within the catalytic valley.

Destabilization of Dcr-2 in the *dcr-2^{A1453T}* mutant affects its downstream function

Our previous study showed that Dcr-2 is also involved in RISC assembly, thereby affecting RISC activity [2]. To determine whether the *dcr-2* missense mutations affect RISC activity, we performed an *in vitro* siRNA-directed, target mRNA cleavage assay (Fig. 2C). Embryo lysates from Dcr-2 helicase domain missense mutants exhibited target mRNA cleavage

activity comparable to wild type, which is consistent with our previous observation that a functional helicase domain is not required for Dcr-2 to mediate its downstream function [2]. Additionally, the *dcr-2^{P1496L}* lysate that was unable to support dsRNA processing exhibited normal RISC activity, confirming that Dcr-2 RNase III activity is not required for RISC-mediated mRNA cleavage [2]. In contrast, RISC activity was greatly impaired in the *dcr-2^{A1453T}* lysate compared with wild type. To examine whether the *dcr-2^{A1453T}* lysate exhibited a defect in RISC assembly comparable to its defective RISC activity, we monitored RISC assembly by native gel electrophoresis (Fig. 2D). The *dcr-2^{A1453T}* lysate showed a significant reduction in holo-RISC formation compared to wild type. Together, these results indicate that the *dcr-2^{A1453T}* mutant is impaired in holo-RISC formation and mRNA cleavage. The Dcr-2/R2D2 complex cooperates to promote loading of siRNAs into RISC [6,7,16]. Dcr-2 stabilizes R2D2, whereas R2D2 is dispensable for the stability of Dcr-2 [16]. Consistent with previous observations, reduced Dcr-2 levels in the *dcr-2^{A1453T}* mutant resulted in less R2D2 protein (Fig. 2B). The down-regulation of both Dcr-2 and R2D2 proteins in the *dcr-2^{A1453T}* mutant did not result from alterations in their mRNA levels. As shown in Fig. 2E, the *dcr-2^{A1453T}* mutant had normal levels of *dcr-2* and *r2d2* mRNAs as determined by real-time RT-PCR analysis. This discrepancy between mRNA levels and protein expression suggests that the Dcr-2 A1453T mutant protein may be unstable. Accordingly, all defects in dsRNA processing, holo-RISC formation, and target mRNA cleavage observed in the *dcr-2^{A1453T}* mutant could be attributable to a decreased abundance of the mutant Dcr-2 protein with the accompanying reduction of R2D2. To examine possible effects of this mutation on Dcr-2 stability, we analyzed the crystal structure of the RNase IIIb domain from human Dicer [15]. As shown in Fig. 3, residue A1686 of human Dicer, which is equivalent to Dcr-2 A1453, is located at the packing interface of the helix-helix interaction between $\alpha 2$ and $\alpha 7$. Since the methyl side chain of the A1686 residue in $\alpha 2$ mediates a hydrophobic interaction with L1815 in $\alpha 7$, the substitution of the A1686 residue by Thr should significantly destabilize the helix-packing interaction. Among Dicer orthologs, the A1686 residue is highly conserved in the RNase IIIb domain, and the L1815 is also conserved as a hydrophobic amino acid (Fig. 1C). Moreover, human Dicer and *Drosophila* Dcr-2 share extensive sequence homologies in the motifs corresponding to helices $\alpha 2$ and $\alpha 7$ (Fig. 1C). Therefore, we suspect that the A1453T mutation of Dcr-2 within the homologous α -helix may perturb packing with a neighboring helix, thus causing misfolding or unfolding of the two helices, leading to the rapid degradation of the mutant Dcr-2 protein. However, since the L1815 residue in $\alpha 7$ is close to the invariant Asp and Glu residues that are involved in coordinating an Mg^{2+} ion at one end of the catalytic valley within the same helix of human Dicer (Fig. 1C), A1453T may also affect the RNase III activity of *Drosophila* Dcr-2, and this effect might destabilize the protein.

The carboxyl-terminal region of R2D2 is essential for binding to Dcr-2

To identify the region of R2D2 that interacts with Dcr-2, we generated four deletion mutants of R2D2 and performed co-immunoprecipitation experiments (Fig. 4). The full-length R2D2 and its deletion derivatives were expressed as FLAG-tagged proteins together with HA-tagged Dcr-2 in S2 cells. HA-tagged EGFP was used as a heterologous control. When the HA-tagged proteins were precipitated with anti-HA antibody, full-length R2D2 co-immunoprecipitated with HA-Dcr-2 but not HA-EGFP. Deletion of the amino-terminal 82 (D1) or 168 (D2) amino acids, thus removing the first or both dsRNA-binding domains (dsRBDs) of R2D2, respectively, did not affect the ability of either variant to interact with HA-Dcr-2. In contrast, the carboxyl-terminal deletion mutants (D3 and D4) of R2D2 failed to interact with HA-Dcr-2. These results indicate that the carboxyl-terminal residues 237–311 of R2D2 are necessary for Dcr-2 binding, whereas the amino-terminal region containing both dsRBDs is dispensable for Dcr-2 interaction.

In conclusion, we characterized six missense mutations in the *dcr-2* gene. Four mutations in the Dcr-2 helicase domain reduced dsRNA processing activity. The P1496L mutation in the RNase IIIb domain of Dcr-2 caused a loss of dsRNA processing activity, suggesting that the Pro residue is critical for the RNase III activity of Dcr-2. The A1453T mutation in the same domain destabilized the Dcr-2 protein, leading to a large reduction in both dsRNA processing and target mRNA cleavage activities. In addition, R2D2 appears to be stabilized by Dcr-2 through its carboxyl-terminal Dcr-2-binding domain. Taken together, our results provide further insight into the structure–function relationship of Dicer, which plays crucial roles in both the initiation and effector phases of the RNAi pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Identification of new *Drosophila dcr-2* missense alleles. (A) The *dcr-2* mutants are defective in eye-specific RNAi against the endogenous *white* gene. Canton *S*, *white*⁺ fly; *GMR-wIR*, *white*⁺ fly with one copy of the *GMR-wIR* transgene which partially silences the *white* gene, resulting in a pale orange eye color; *dcr-2*^{L811fsX}, *white*⁺ fly with one copy of *GMR-wIR* and homozygous for a null allele of *dcr-2*, exhibiting strong suppression of *white* RNAi. The *white*⁺ flies with one copy of *GMR-wIR* and homozygous for each missense allele of *dcr-2* in this study are indicated. The *dcr-2* missense mutants exhibit stronger eye pigmentation relative to the *GMR-wIR* fly. (B) A schematic representation of the domain structure of Dcr-2. Indicated are the DExH-box (HN)- and C-helicase (HC) domains; a domain of unknown function (DUF) 283; the tandem repeats of the RNase III domain (RIIIa and RIIIb); and a dsRNA-binding domain (RBD). The positions of the *dcr-2* missense mutations identified in this study are indicated below the domain structure. (C) Multiple alignment of the RNase IIIb domains of Dicers from human (Hs-Dcr), *Drosophila* (Dm-Dcr-1 and Dm-Dcr-2), *C. elegans* (Ce-Dcr), *S. pombe* (Sp-Dcr), and *Giardia* (Gi-Dcr) and the corresponding domains of RNase III enzymes from *Aquifex* (Aa-RNase III) and *E. coli* (Ec-RNase III). Alignments were performed using ClustalW. Numbers on the left represent amino acid sequence numbering. Invariant residues are shaded in pink and conserved residues are shaded in blue (100% conservation) and gray (75% conservation). The *dcr-2* missense mutations are indicated by triangles. Invariant acidic residues that coordinate a divalent metal ion are marked with asterisks. Based on the crystal structure of the RNase IIIb domain from human Dicer, the secondary structure (α -helix) is shown above the alignment. The RNA-binding motif RBM 3 in Aa-RNase III is boxed in red. A residue in helix α 7 that makes a hydrophobic interaction with the residue, corresponding to Dcr-2 A1453, in helix α 2 of the RNase IIIb domain of human Dicer is indicated by an arrow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Biochemical characterization of *dcr-2* missense mutants. (A) *In vitro* dsRNA processing assay. Processing of siRNAs from internally labeled *Photinus pyralis* luciferase (*Pp-luc*) dsRNA substrate was examined with lysates from either wild type or *dcr-2* mutant embryos. Shown are the labeled siRNA products of each reaction. No lysate, reaction with lysis buffer in place of embryo lysate. (B) Western blot analysis for Dcr-2 and R2D2 protein levels in either wild type or *dcr-2* mutant embryos. Anti- β -tubulin antibody was used to verify equal loading of protein on the gel. The weak signal corresponding to R2D2 in lane *dcr-2^{L81fsX}* originates from a protein cross-reacting with anti-R2D2 antibody. (C) *In vitro* target mRNA cleavage assay. Targeted cleavage of 5'-labeled *Pp-luc* mRNA by a complementary synthetic siRNA was examined with lysates from wild type or *dcr-2* mutant embryos. The 5'-labeled *Pp-luc* mRNA and the 5' cleavage product are indicated by an arrow and arrowhead, respectively. Input, unreacted *Pp-luc* mRNA; No siRNA, reaction without *Pp-luc* siRNA. (D) *In vitro* RISC assembly assay. Labeled *Pp-luc* siRNA was incubated with lysates from wild type or *dcr-2* mutant embryos, and siRNA-protein complexes as indicated were resolved by native gel electrophoresis. RDI (R2D2/Dcr-2 initiator) and RLC (RISC-loading complex) are precursor complexes for holo-RISC. An asterisk indicates material too large to be resolved in the gel. (E) Real-time RT-PCR (left) and RT-PCR (right) analyses of *dcr-2* and *r2d2* mRNA levels in wild type or *dcr-2^{A1453T}* embryos. For the real-time RT-PCR experiment, the levels of *dcr-2* and *r2d2* mRNAs normalized to the corresponding level of *RP49* are shown in triplicate along with standard errors.

Fig. 3.

Ribbon diagram of the RNase IIIb domain of human Dicer. The methyl side chain of A1686 in helix $\alpha 2$ is indicated by a yellow ball. The right panel highlights the close helix-packing interaction between helices $\alpha 2$ and $\alpha 7$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Mapping of the Dcr-2-interacting domain in R2D2. (A) Schematic representation of FLAG-tagged R2D2 (WT) and its deletion derivatives (D1–D4). Numbers in parentheses denote amino acid positions. (B) Co-immunoprecipitation (co-IP) assays. Expression plasmids harboring epitope-tagged genes were transfected into S2 cells as indicated. HA-tagged EGFP was used as a heterologous control. After induction of expression for 48 h, whole-cell lysates were prepared, and either directly blotted (left) or immunoprecipitated with anti-HA antibodies conjugated to beads before blotting (right). Western blots (WB) were performed with anti-HA and anti-FLAG antibodies as indicated. Tagged proteins in the blots are indicated to the right, and asterisks mark the anti-HA immunoglobulin G light chains.