Evolutionarily Conserved Roles of the Dicer Helicase Domain in Regulating RNA Interference Processing*

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Background: Dicer is a ribonuclease required for microRNA biogenesis.

Results: The two related Dicer enzymes from the thermophilic fungus Sporotrichum thermophile have distinct functions in RNA processing.

Conclusion: The helicase domains from each Dicer define the RNA substrate specificity and have distinct RNA binding and ATP hydrolytic activities.

Significance: A regulatory function for the helicase domain is conserved from fungi to humans.

The enzyme Dicer generates 21–25 nucleotide RNAs that target specific mRNAs for silencing during RNA interference and related pathways. Although their active sites and RNA binding regions are functionally conserved, the helicase domains have distinct activities in the context of different Dicer enzymes. To examine the evolutionary origins of Dicer helicase functions, we investigated two related Dicer enzymes from the thermophilic fungus Sporotrichum thermophile. RNA cleavage assays showed that S. thermophile Dicer-1 (StDicer-1) can process hairpin precursor microRNAs, whereas StDicer-2 can only cleave linear double-stranded RNAs. Furthermore, only StDicer-2 possesses robust ATP hydrolytic activity in the presence of double-stranded RNA. Deletion of the StDicer-2 helicase domain increases both StDicer-2 cleavage activity and affinity for hairpin RNA. Notably, both StDicer-1 and StDicer-2 could complement the distantly related yeast Schizosaccharomyces pombe lacking its endogenous Dicer gene but only in their full-length forms, underscoring the importance of the helicase domain. These results suggest an in vivo regulatory function for the helicase domain that may be conserved from fungi to humans.

siRNAs are formed from two separate RNA strands that comprise perfectly base-paired duplexes (2). Once processed by Dicer, both miRNAs and siRNAs function as sequence-specific guides to recruit Argonaute proteins and associated factors to complementary mRNAs for post-transcriptional silencing of gene expression (3, 4). Although RNAi is conserved among many eukaryotes, multiple duplications of its protein components have expanded the diversity and complexity of the pathway (5, 6). Many species express multiple Dicer proteins, and it is often unclear whether these copies have functionally distinct or overlapping roles in RNAi. Here, we have investigated two fungal Dicers from Sporotrichum thermophile and show that their different functions are likely due to changes in their helicase domains.

Canonical Dicer consists of an N-terminal DEX(D/H) box RNA helicase, a DUF283 domain, a PAZ domain, tandem RNase III domains, and a C-terminal double-stranded RNA-binding domain (Fig. 1A). Previous biochemical and structural studies show that the size of product RNAs is determined by the spatial arrangement of the PAZ and RNase III domains, as well as the binding pocket for the RNA 5’ end (7, 8). The RIG-I-like helicase domain, which is located adjacent to the RNase III domains, has emerged as a substrate specificity determinant and may have developed specialized roles for different Dicer proteins.

Mammals and nematodes have only one Dicer enzyme, and differences in the cleavage rates of pre-miRNA and pre-siRNA substrates are attributable to the helicase domain (9, 10). Human Dicer cleaves pre-miRNAs much more efficiently than pre-siRNAs, with the helicase domain inhibiting siRNA production. In Drosophila melanogaster, which contains two Dicers, processing of these two types of RNA substrates is segregated such that Dicer-1 is responsible for generating miRNAs, and Dicer-2 generates siRNAs (11). Dicer-1 contains an inactive helicase domain that is unable to hydrolyze ATP, which facilitates binding to the loops of pre-miRNAs (12), whereas Dicer-2 has an active helicase that enables processive cleavage of long dsRNAs (13–15). The helicase domain may have allowed Dicer to adapt rapidly to a diverse array of substrates.

The abbreviations used are: dsRNA, double-stranded RNA; miRNA, microRNA; siRNA, small interfering RNA; StDicer, S. thermophile Dicer-1; HsDicer, H. sapiens dicer; DmDicer, D. melanogaster dicer; NcDicer, Neurospora crassa dicer; Sp, Schizosaccharomyces pombe dicer; TBZ, thiabendazole; ΔHel, Dicer without helicase domain; HD, helicase domain; ssRNA, single-stranded RNA; nt, nucleotide; smloop, small loop; TEV, tobacco etch virus; qPCR, quantitative PCR.

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TABLE 1
 Sequences of oligonucleotides

| Oligonucleotide sequence | Hairpin | smlloop hairpin | pStem-a hairpin | pStem-b hairpin | 37a RNA | 37b RNA | 37b DNA | 27a RNA | 27b RNA | 24a RNA | 24b RNA | 53a DNA | 53b DNA |
|--------------------------|---------|-----------------|-----------------|-----------------|----------|---------|---------|---------|---------|---------|---------|---------|---------|
|                          | 5′-UGA GGU AGU AGG UUG UAU AGU UUU AGG GUC ACA CCC ACC ACU GGG AGA UAA CUA UAC AAA CUA CUG UCU UAC C-3′ | 5′-UGA CUC UUC AGA AGG UUG ACU GGU GAA UCU CAU GGC AAC ACC AGU CUA UGG GCU GUC-3′ | 5′-UGA GGU AGU AGG UUG UAU AGU UUU AGG GUC ACA CCC ACC-3′ | 5′-P-ACU GGG AGA UUC AAA CUA UAC AAC CUA CUA CCA AA-3′ | 5′-TGG TGA ACT TTC AAA CTA TAC AAC CTA CTA CCT CAT T-3′ | 5′-TG GCG CAG CUG CCC AAG UCU CUG CUG AAA-3′ | 5′-TG CCA AGC UGU AGC ACA ACT TG TGG TAT CAA TAG TTG TAA TGT ACG TAT AC-3′ | 5′-GTA CAG CTA CAT TAC AAT CAT TGA TAG CAT CAA TGG CTG CTT CTA ACT CCA TC-3′ |

Insight into the function of Dicer in other organisms could shed light on the evolution of this important enzyme.

An obstacle to studying Dicers biochemically has been their large sizes and difficulty in purification, so we turned to the thermophilic fungus *Sporotrichum thermophile* as a system well suited to purification of its two stable Dicers, StDicer-1 and StDicer-2. We discovered different biochemical activities for the isolated helicase domain of each of these proteins that could in turn influence the function of the full-length enzyme. The activities of these two Dicers are reminiscent of the RNAi pathway in *D. melanogaster*, highlighting how the helicase domain evolved as a conserved regulator of small RNA processing in eukaryotes.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—The genes encoding *stdicer-1* (Q2H0G2.2) and *stdicer-2* (XP_001228335.1) were amplified from the genomic DNA of *S. thermophile*, and the introns were removed using site-directed mutagenesis PCR. The full-length coding sequences as well as sequences encoding truncated proteins lacking the helicase domain (ΔHel) were cloned into a customized pFastBac expression vector (4C, Addgene 30116) using ligation-independent cloning, resulting in protein expression constructs that were fused downstream of a hexahistidine maltose-binding protein tag and a TEV protease. Proteins were further purified by size-exclusion chromatography using a Superdex 200 (16/60) column (GE Healthcare) in purification buffer.

The helicase domains from *stdicer-1* and *stdicer-2* were cloned into a customized pET expression vector (1M, Addgene 29656) using ligation-independent cloning, resulting in protein expression constructs that were fused downstream of a His$_6$ maltose-binding protein tag and a TEV protease cleavage site. The plasmids were transformed into BL21(DE3) cells, and the proteins were purified using the protocol described above, except that the final size-exclusion chromatography was performed using a Superdex 75 (16/60) column (GE Healthcare). All purification steps were carried out at 4°C. All protein concentrations were determined using a NanoDrop (Thermo).

**DNA and RNA Substrates**—All of the DNA and RNA substrates used, with the exception of the hairpin pre-miRNA substrate, were synthesized by Integrated DNA Technologies. The sequence of the hairpin RNA substrate was derived from human pre-let-7a-1, and it was synthesized by Integrated DNA Technologies. The sequence of the hairpin RNA substrate was derived from human pre-let-7a-1, and it was synthesized by Integrated DNA Technologies. The sequence of the hairpin RNA substrate was derived from human pre-let-7a-1, and it was synthesized by Integrated DNA Technologies. The sequence of the hairpin RNA substrate was derived from human pre-let-7a-1, and it was synthesized by Integrated DNA Technologies. The sequence of the hairpin RNA substrate was derived from human pre-let-7a-1, and it was synthesized by Integrated DNA Technologies. The sequence of the hairpin RNA substrate was derived from human pre-let-7a-1, and it was synthesized by Integrated DNA Technologies. The sequence of the hairpin RNA substrate was derived from human pre-let-7a-1, and it was synthesized by Integrated DNA Technologies. The sequence of the hairpin RNA substrate was derived from human pre-let-7a-1, and it was synthesized by Integrated DNA Technologies.

The RNA oligonucleotide 37a RNA can form a perfectly matched duplex containing a 2 nt 3′ overhang with either 37b RNA (35-bp dsRNA) or 37b DNA (35-bp DNA/RNA heteroduplex). The RNA oligonucleotide 27a can hybridize with 27b (25-bp dsRNA) and 24a can hybridize with 24b (22-bp dsRNA), both with 2-nt 3′ overhangs. The DNA oligonucleotides 53a DNA and 53b DNA form a completely complementary duplex (53-bp dsDNA). The sequences of all of substrates used in this study are in Table 1.
Mechanism of Fungal Dicers

For the dicing assays and electrophoretic mobility shift assays (EMSSAs), the substrates were 5'-end-labeled with T4 polynucleotide kinase (New England Biolabs, Inc., Beverly, MA) and [α-32P]ATP (PerkinElmer Life Sciences), gel-purified, and annealed before use. Annealing was performed in 100 mM Tris-HCl (pH 7.5), 3 mM MgCl₂, and 30 mM NaCl by heating at 95 °C for 5 min and either slow cooling (perfect duplex RNAs) or flash cooling (hairpin RNAs).

Dicing Assays—Labeled and annealed RNA substrates were incubated with 1 μM StDicer at 37 °C for the specified time in a 10-μl reaction volume containing 20 mM Tris-HCl (pH 6.5), 5 mM MgCl₂, 100 mM NaCl, 1 mM DTT, and 1% glycerol. 1 mM ATP was added to reactions with ATP. Reactions were stopped by addition of 1.2 volumes of loading buffer (95% formamide, 50 mM EDTA, 0.025% SDS, 0.1% xylene cyanol FF, and 0.1% bromophenol blue). After heating at 95 °C for 5 min, the samples were analyzed by electrophoresis with a 12.5% polyacrylamide, 7 M urea gel run in 0.5× TBE buffer and quantified using the PhosphorImager/ImageQuant (GE Healthcare).

ATP Hydrolysis Assay—Protein (1 μM) was incubated with 1 mM ATP, 10–100 nM [α-32P]ATP (3000 Ci/mmol, PerkinElmer Life Sciences), and 4 mM nucleic acid substrate in a buffer consisting of 20 mM Tris-HCl (pH 6.5), 5 mM MgCl₂, 25 mM NaCl, 1 mM DTT, and 1% glycerol. Reactions were stopped with the addition of 100 mM EDTA, spotted onto 20 × 20 cm PEI Celulose F plates (EMD Millipore), and chromatographed in 1 M formic acid and 0.5 M LiCl until the solvent traveled ¾ of the plate. The plate was dried and quantified using the PhosphorImager/ImageQuant (GE Healthcare).

Electrophoretic Mobility Shift Assay—Approximately 0.5–1 nm (500–1000 cpm) labeled and annealed RNA substrates were incubated with the indicated concentrations of StDicer constructs for 30 min at 4 °C in 20 mM Tris-HCl (pH 7.5), 25 mM NaCl, 5 mM EDTA, 1 mM DTT, and 1% glycerol. 1:2 serial dilutions were used to reach the indicated concentrations. Reactions were analyzed on a 6% native polyacrylamide gel and quantified using the PhosphorImager/ImageQuant. Percent bound RNA was plotted as a function of protein concentration. Kᵅ value was determined by global fitting to the equation fraction bound = A × [protein]/(Kᵅ + [protein]), where A is the amplitude of the binding curve. Curve fitting was conducted with KaleidaGraph (Synergy Software, Reading, PA).

S. pombe Strains, Media, and Constructs—The S. pombe dcr1 gene was amplified from genomic DNA. All proteins were cloned into the pREP1 vector, carrying a leu selection marker and nmt1 promoter, using Ndel and BamHI. The S. pombe strain h− Δdcr1+::kanMX6 ura4-D18 leu1−32 was used for functional complementation assays (8). Transformed cells were grown in minimal media with glutamate as the nitrogen source (PMG, Sunrise Science), supplemented with 225 mg/liter adenine, histidine, lysine hydrochloride, and uracil. Once the cells reached an A₆₀₀ of 0.4, cells were either plated or grown in liquid culture containing yeast extract media (YES or rich media) or modified KsnoT media with 15 μg/ml thiabendazole (Santa Cruz Biotechnology) (17). Plates were imaged after about 4 days of growth at 30 °C. Liquid culture measurements were done in 96-well plates using a plate reader (Tecan Infinite F200 Pro). The liquid cultures were fit to either an exponential growth equation using Prism or a logistic growth equation using the software programming language R.

qRT-PCR—RNA was purified from 1 ml of saturated Schizosaccharomyces pombe cultures using hot acid phenol and chloroform. Residual DNA was removed by DNase treatment (Promega), after which the RNA was purified again by ethanol precipitation. cDNAs were prepared with an Invitrogen Superscript III kit followed by the addition of RNase H (New England Biolabs). cDNAs were quantified with a Stratagene MX3000 quantitative PCR system using DyNAmo HS SYBR Green (Thermo). All primer set amplification values were normalized to ACTI amplification values. All primer sets used can be viewed in Table 2.

### RESULTS

StDicer-1 and StDicer-2 Display Distinct Cleavage Preferences—We performed a phylogenetic analysis to search for smaller eukaryotic Dicers that might be tractable for biochemical investigation, leading to selection of the thermophilic fungus, S. thermophile (synonymous with Myceliophthora thermo philophila) (18). This organism possesses two Dicers containing the domains that are common to most eukaryotic Dicers (Fig. 1A). In particular, both the helicase and the tandem RNase III domains are well conserved and contain the putative metal-coordinating residues required for enzymatic activity (Fig. 1B). Both proteins are significantly smaller than human Dicer-1 due to two large deletions. One deletion occurs between the putative PAZ domain and the first RNase III domain, and the second is within the first RNase III domain (Fig. 1, A and B). Both of these regions are poorly conserved among eukaryotic Dicers, and the latter deletion in the RNase IIIa domain is proposed to be an Argonaute-binding site unique to vertebrates (19). However, we were unsure whether these deletions would affect the function of these Dicers and proceeded to investigate their catalytic activities.

We first assayed RNA cleavage by incubating purified StDicer-1 and StDicer-2 with two different radiolabeled RNAs in the presence or absence of ATP (Fig. 2, A and B). Because it was difficult to predict computationally the natural S. thermo philophila Dicer substrates due to poor sequence and structure conservation (20), we first used two RNA substrates that have been well characterized for other eukaryotic Dicers (Table 1) (9, 10). One substrate, 35-bp RNA, mimics a pre-siRNA substrate and includes two 37-nt RNA strands that hybridize to form a perfect complement.
RNA duplex with 2 nt, 3' overhangs. The other substrate mimics a pre-miRNA hairpin and is derived from human pre-let7, containing an imperfectly base-paired stem and a 27-nt loop. RNA cleavage was observed with both proteins, but only StDicer-1 was able to cleave the hairpin RNA to 25 nt, which is a length consistent with possible loading into Argonaute and is the observed cleavage length for small RNAs in fungal RNAi (20, 21). The rate of RNA cleavage for StDicer-1 with the hairpin RNA substrate was the same in the presence and absence of ATP (Fig. 2).

StDicer-2 was only able to generate 25-nt products from the 35-bp pre-siRNA substrate (Fig. 2, A and B). However, the rate of cleavage was slower than that observed for StDicer-1 with the 35-bp pre-siRNA. Notably, we observed robust hairpin cleavage activity of StDicer-2, but it did not generate a 22–27-nt product that would be likely to function as a guide strand within the Argonaute for downstream silencing. Cleavage activity of StDicer-2 was unaffected by ATP, a behavior distinct from that of D. melanogaster Dicer-2 (13, 14), suggesting that additional factors may be required for efficient RNA cleavage.

**StDicer-1 Is a Thermostable Protein That Cleaves Pre-miRNAs**—To further investigate the different RNA cleavage activities of StDicer-1 and StDicer-2, we tested additional pre-miRNA substrates and temperatures (Fig. 2, C–E) (22, 23). The two RNA substrates tested were a pre-miRNA with a reduced loop size of 5 nt (smloop) and a related hairpin with a large loop but a completely perfect RNA stem hairpin. Both RNAs were rapidly cleaved by StDicer-1 and not by StDicer-2 (Fig. 2C).

This suggests that the loop of the pre-miRNA may inhibit cleavage by StDicer-2 as none of the substrates could be cleaved regardless of the loop size. Alternatively, the RNA substrate may need to have a perfectly base-paired duplex longer than 25 bp to allow for StDicer-2 cleavage as the perfect stem hairpin had a 25-bp stem. Because StDicer-1 more efficiently and accurately cleaves RNA, the temperature-dependent cleavage was tested for the smloop pre-miRNA and compared with human Dicer (HsDicer). Four different temperatures were assayed as follows: 30 °C, the optimal temperature for S. pombe growth; 37 °C, the temperature at which all other assays were performed; 48 °C, the optimal temperature for S. thermophile growth; and 68 °C. Although both enzymes were able to cleave the pre-miRNA at 30 °C, StDicer-1 was significantly more thermostable than StDicer-2, with optimal activity at 68 °C.

**Mechanism of Fungal Dicers**

FIGURE 1. Domain architecture and alignment of StDicer-1 and StDicer-2 with other eukaryotic Dicers. A, schematic representation of the domain organization and phylogenetic relationship of StDicer-1 and StDicer-2 to other eukaryotic Dicers. The left side of the panel is the phylogenetic relationship between Dicers from S. thermophile (StDicer) and Dicers from humans (HsDicer), D. melanogaster (DmDicer), S. pombe (SpDicer). On the right side of the panel is the domain architecture of human and StDicer. The helicase domain is organized into three lobes termed HEL1, HEL2l, and HEL2. The gray dashed lines represented large regions of HsDicer that are absent in StDicer. B, multiple sequence alignment output from ClustalX comparing the amino acid sequence of S. thermophile dicer-1 (St_Dicer1) and dicer-2 (St_Dicer2), which are outlined in red, and closely related proteins from human (Hs_Dicer), D. melanogaster (Dm_Dicer1, Dm_Dicer2), N. crassa (Nc_Dicer1, Nc_Dicer2), S. pombe (Sp_Dicer), and Giardia intestinalis (Gi_Dicer). Dark blue represents positions that have a single, fully conserved residue with the two lighter blue colors indicating strongly conserved and weakly conserved residues. The helicase domain is one of the most conserved regions among full-length Dicers. The highlighted motifs are involved in binding an NTP, typically ATP, and the energy of hydrolysis is used to dynamically interact with RNA. S. thermophile Dicers contain intact Rnase III domains and the residues highlighted by the red asterisks are involved in coordinating Mg²⁺ in the G. intestinalis structure.
efficiently at the highest temperature, HsDicer’s cleavage rate plateaued at 37 °C, whereas the rate of cleavage for the thermophilic protein continued to increase (Fig. 2, D and E). This indicates that as anticipated, the thermophilic proteins may be more stable at the higher temperatures.

**Mechanism of Fungal Dicers**

We first investigated which nucleic acid species stimulated activity for full-length protein StDicer-2 (Fig. 3B). We incubated the protein with single- or double-stranded DNA or RNA as well as a DNA/RNA heteroduplex. The slight stimulation of activity was once again observed in the presence of ssRNA. ATP hydrolysis further increased in the presence of an A-form duplex such as the DNA/RNA heteroduplex and reached a maximum with dsRNA.

With *D. melanogaster* Dicer-2, the rate of ATP hydrolysis for dsRNA substrates of different lengths was comparable (13). In contrast, significant differences between the different nucleic acid substrates observed for *S. thermophile* ATPase activity led us to test whether changes in the RNA structure could alter the
rate of ATP hydrolysis (Fig. 3C). We incubated StDicer-2 with four different RNA substrates. Three of the RNAs were perfect RNA duplexes that ranged in size from 35 to 22 bp, representing Dicer substrates and products, respectively. The fourth RNA substrate was a hairpin RNA containing an imperfect 22-bp stem and a 27-nt loop. There was no significant difference in the rates of ATP hydrolysis, similar to what was observed with *D. melanogaster* Dicer-2 (13).

We finally isolated the helicase domain (HD) from both StDicer-1 and StDicer-2 and measured ATP hydrolysis rates in the presence and absence of nucleic acid substrates (Fig. 3D). StDicer-1 HD displayed no activity even in the presence of 35-bp RNA; similar results were obtained for full-length StDicer-1. In contrast, StDicer-2 HD showed RNA-dependent ATP hydrolytic activity. Although both ssRNA and dsRNA stimulated ATP hydrolysis by StDicer-2 HD, a significantly higher increase was observed in the presence of dsRNA. These results are similar to those obtained for other RIG-I family helicases, of which Dicer is a member, for which ATP hydrolysis is only observed when the protein is bound to nucleic acid (13, 24).

**RNA Binding Properties of StDicers**—In light of the observed differences in cleavage activity, we investigated the binding affinities of corresponding RNA substrates. Affinities were measured by EMSA with 0.5–1 nM RNA substrate and increasing protein concentrations (Fig. 4 and Table 3). StDicer-1 had higher affinity for both hairpin and dsRNA substrates, in agreement with its observed rapid rates of cleavage. StDicer-2 had significantly weaker affinity to the RNA substrates tested, with a slightly higher affinity for duplex RNA substrates (Fig. 4B).

The observation that the two StDicers have different RNA binding and cleavage activities led us to test the RNA binding affinities of the isolated HD from StDicer-1 and StDicer-2 (Fig. 4C). Although weaker than that observed for the full-length proteins, the measured affinities followed a similar trend. StDicer-1 HD has the highest affinity, with a 100-fold difference in affinity between hairpin and duplex RNA substrates. StDicer-2 had only a 2-fold difference for duplex RNA substrates over hairpin RNAs, which was ~1–10 μM, with a slightly higher affinity for duplex RNA substrates (Fig. 4B).

**Mechanism of Fungal Dicers**

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These results indicate that the helicase domain inhibits pre-miRNA binding.

In accordance with the RNA binding data, removal of the helicase domain greatly increased the rates at which some RNA molecules were cleaved. This protein showed increased cleavage rates with the duplex RNA substrates (Fig. 5B). Surprisingly, although the affinity for hairpin RNA substrates increased greatly, the protein was still unable to cleave these RNA molecules to produce miRNA length products. As with the full-length proteins, the addition of ATP did not change the rate of RNA cleavage. These results are consistent with studies of *Drosophila* and human Dicers and indicate there may be a universal mode of regulation that involves the helicase domain (9, 13). However, the catalytic core still retains some selectivity for RNA processing.

**TABLE 3**

| Kd (nM) | StDicer-1 | StDicer-2 | StDicer-1 HD | StDicer-2 HD |
|---------|-----------|-----------|-------------|-------------|
| 35-bp duplex | 76 ± 14 | 1800 ± 100 | >10,000 | 3200 ± 400 |
| Hairpin RNA | 33 ± 3 | 9600 ± 300 | 930 ± 80 | 6500 ± 2000 |
| ΔHel StDicer-2 | 900 ± 200 | 9300 ± 30 |

These results indicate that the helicase domain inhibits pre-miRNA binding.

To determine whether the helicase domain is functional in *S. pombe*, we used a genetic complementation assay in *S. pombe*, which has been used successfully before to assay the function of human and *Giardia* Dicer (8, 25). *S. pombe* only contains one endogenous Dicer (SpDicer) and strains lacking Dicer (KO) exhibit slight growth impairment. In *S. pombe*, RNAi is needed for the production of small RNAs that are generated from the centromere and are necessary for proper chromosome segregation and microtubule attachment during mitosis (25). These mitotic defects can be exacerbated by the addition of the microtubule-destabilizing drug thiabendazole (TBZ), nearly preventing the growth of the KO strains (25).

This KO strain was transformed with different Dicer constructs, and growth was assayed by plating serial dilutions on rich media or media containing TBZ (Fig. 6A). On rich media, the growth of strains containing full-length Dicer from *S. pombe* or *S. thermophile* was nearly indistinguishable from wild-type and empty vector growth. Surprisingly, the strains expressing the StDicer constructs lacking the helicase domain were quite sick and showed decreased growth on rich media. The mRNAs encoding the episomally expressed Dicer proteins were all at least 3-fold higher than the wild-type Dicer expression as determined by RT–qPCR (Fig. 6, B and C). This, gene expression levels of these constructs are likely to be similar. Previously, it has been shown that Giardia Dicer (8), which
lacks the helicase domain altogether, is able to partially complement the KO strain. We propose that the helicase domains of the StDicer enzymes help to restrict the access of small RNAs to the protein, preventing promiscuous RNA binding and cleavage. It is possible that the *Giardia* Dicer has evolved to not require this additional inhibition and has other ways to regulate substrate cleavage.

On media containing TBZ, only strains containing full-length Dicer were able to suppress the growth defects of the endogenous Dicer deletion. We noted that there was a slight difference in growth between strains expressing StDicer-1 versus StDicer-2, so we assayed the growth in liquid culture to quantify the changes in growth (Fig. 6D). By fitting the growth data of StDicer-1 and StDicer-2 to exponential curves, we observed that the expression of StDicer-1 gave an ~2-fold increase in growth ($t_d = 12.3 \pm 0.4$ h) over strains containing StDicer-2 ($t_d = 22.3 \pm 0.7$ h). Although both of these growth behaviors were significantly faster than that observed for the empty vector control, they were only a fraction of the growth rate observed for wild-type *S. pombe* ($t_d = 1.7 \pm 0.1$ h) and KO complemented with SpDicer ($t_d = 1.6 \pm 0.1$ h) when fit with a logistic growth equation. These results indicate that full-length Dicers from *S. thermophile* are functional orthologs to the previously described Dicer systems and can maintain RNAi in a heterologous system.

**DISCUSSION**

Dicer is a large, multifunctional protein, and previous biochemical studies have focused on how its noncatalytic domains affect RNA cleavage. We have found that the helicase domain plays an evolutionarily conserved role in selecting RNAs for Dicer-mediated cleavage. Because of the technical challenges in purifying fragments from human Dicer, we turned to the thermophilic fungus *S. thermophile*. We found that distinct biochemical activities of the isolated helicase domains from both Dicers contribute to the specialized functions of the full-length proteins. This provides insight into its evolution as a core enzyme of the RNAi machinery.

Conflicting models have emerged for the function of these two Dicers in fungi. A previous study on *Neurospora crassa* indicated that these proteins are redundant in function during quelling, a process in which long dsRNAs lead to decreased...

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**FIGURE 5. Removal of the helicase domain increases RNA affinity and the rate of RNA cleavage.** A, removal of the helicase domain for StDicer-2 greatly increases the affinity for RNAs. The affinity for two different RNAs, 35-bp and hairpin RNA, was measured for ΔHel StDicer-2 (ΔHel2). The data for StDicer-1 (1) and StDicer-2 (2) are reproduced from Fig. 4B for comparison. B, removal of the helicase domain increases the rate of RNA cleavage. The fraction of RNA cleaved is plotted for a 35-bp RNA with and without the presence of ATP and the graphs for the lines overlap. The data for StDicer-1 (1) and StDicer-2 (2) are reproduced from Fig. 2B for comparison.
FIGURE 6. StDicer-1 and StDicer-2 complement growth defects in vivo. A, overexpression of StDicer-1 and StDicer-2 rescues the TBZ sensitivity of the S. pombe Dicer delete (KO). Knock-out cells were transformed with a vector expressing the indicated proteins and grown at 30 °C. Growth was assayed by spotting 5-fold serial dilutions of the cultures indicated and plating on nonselective medium (NS) or medium supplemented with 15 μg/ml TBZ. B, relative gene expression by RT-qPCR. Primers from Table 2 were used to quantify the relative levels of mRNA transcript that was either expressed from the plasmid or endogenously expressed. The gene-specific primers were used for each StDicer construct, and SpDicer primers were used for KO/empty vector, KO/SpDicer, and WT. All values were normalized to ACT levels, and the standard deviations were derived from three replicates. C, PCR products from RT-qPCR. The products from the RT-qPCR were run on a 1.5% agarose gel, and the correct sizes were obtained for all reactions except for the empty vector control. The products obtained for the empty vector control were nonspecific products from qPCR as verified by post-amplification melting-curve analysis and agarose gel electrophoresis. In addition, these levels are 10-fold less than those obtained from the WT control. D, knock-out cells expressing StDicer-1 have higher growth rates than those expressing StDicer-2. Cells were grown in liquid culture containing 15 μg/ml TBZ, and time points were taken every 1.5 h for 30 h. Error bars for each time point represent the standard deviation from three replicates.
expression of corresponding genes (26). However, another study involving the filamentous fungus *Magnaporthe oryzae* showed that only one Dicer isoform is responsible for siRNA accumulation (27). Our biochemical and *S. pombe* complementation data support the hypothesis that the two proteins are not redundant in the small RNA pathway. RNA cleavage assays showed that although both proteins can cleave duplex RNAs, StDicer-2 produced more homogeneous siRNA products than those generated by StDicer-1. The differences in the two proteins are more pronounced when testing for pre-miRNA processing, where only StDicer-1 was able to cleave hairpin RNAs. Only recently have functional miRNA-like mechanisms been discovered in fungi, and the distinctions between individual Dicer protein have yet to be fully elucidated in the processing of hairpin RNAs (20).

The distinct functions for eukaryotic Dicer can be attributed to the different activities of the helicase domain. Recent studies have shown that the helicase domain, which cannot hydrolyze ATP, preferentially interacts with the loops of pre-miRNAs and inhibit cleavage of pre-siRNAs (12, 22). This is supported by structural studies with human Dicer, which has an inactive helicase domain, demonstrating that pre-siRNAs are held in a nonproductive conformation between the PAZ and helicase domain (28). In addition, pre-miRNAs induce structural changes involving the helicase domain of some Dicers that enable productive substrate recognition and rapid cleavage (22, 28). Our ATP hydrolysis assays and RNA binding experiments with StDicer-1 support this hypothesis, as only StDicer-1, which bears an inactive helicase domain, could cleave pre-miRNAs.

In contrast, the active helicase domains of other Dicers are found to be important to generate multiple siRNAs processively from a long dsRNA precursor. This has been shown with *D. melanogaster* Dicer-2, where mutations to the helicase domain abolish RNA cleavage in vivo (11). Because *Drosophila* has two Dicer proteins, one hypothesis for the divergent function of Dicer-2 in flies is that this protein, which is one of the fastest evolving genes in the *Drosophila* genome, became a component of insect-specific antiviral defense within flies (29, 30). An emerging alternative view holds that Dicer was duplicated much earlier in metazoan evolution, and one copy was subsequently lost as alternative antiviral defenses were developed (31). Our results support the latter hypothesis, although we cannot rule out the possibility that the activity of StDicer-2 could have arisen by convergent evolution.

Although we were not able to explore the ΔHel StDicer-1 truncation as we did for ΔHel StDicer-2 due to poor expression and solubility, similar truncations have been made for *D. melanogaster* Dicer-1 (DmDicer-1) and HsDicer. For DmDicer-1, the *Kₐ* value for a pre-let-7 substrate increased from 25 to 121 nM once the helicase domain was removed (12). A similar trend was observed for HsDicer, where the *Kₐ* value for the ΔHel truncation increased for both dsRNA and pre-hlet-7 (9). In contrast, with StDicer-2, removal of the helicase domain decreased the *Kₐ* values for both RNA substrates. These data are consistent with the hypothesis that the active helicase domain in one context inhibits RNA binding for StDicer-2, although an inactive helicase domain facilitates RNA binding for DmDicer-1 and HsDicer.

It is interesting to consider how Dicers with an active helicase domain may have evolved different modes of RNA recognition that promote interaction with dsRNA and more facile access to the catalytic center. The closest structure is that from RIG-I, and the preferential binding of dsRNA is likely conserved with StDicer-2 but not with StDicer-1 (32–35). Understanding the detailed molecular changes between these two enzymes could provide further insight into how Dicer recognizes RNAs and how this helicase family has evolved to enable substrate selection. Aside from providing evolutionary insights, Dicers from a thermophilic fungus also exhibit increased stability over other eukaryotic Dicers, making them a great model system for further functional and structural studies of proteins in this family.

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