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Substrate-specific kinetics of Dicer-catalyzed RNA Processing

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Summary

The specialized ribonuclease Dicer plays a central role in eukaryotic gene expression by producing small regulatory RNAs – miRNAs and siRNAs – from larger double stranded RNA (dsRNA) substrates. Although Dicer will cleave both imperfectly base-paired hairpin structures (pre-miRNAs) and perfect duplexes (pre-siRNAs) *in vitro*, it has not been clear whether these are mechanistically equivalent substrates and how dsRNA binding proteins such as TRBP influence substrate selection and RNA processing efficiency. We show here that human Dicer is much faster at processing a pre-miRNA substrate compared to a pre-siRNA substrate under both single and multiple turnover conditions. Maximal cleavage rates (V_{max}) calculated by Michaelis-Menten analysis differed by more than 100-fold under multiple turnover conditions. TRBP was found to enhance dicing of both substrates to similar extents, and this stimulation required the two N-terminal dsRNA binding domains of TRBP. These results demonstrate that multiple factors influence dicing kinetics. While TRBP stimulates dicing by enhancing the stability of Dicer-substrate complexes, Dicer itself generates product RNAs at rates determined at least in part by the structural properties of the substrate.

Introduction

MicroRNAs (miRNAs) and short interfering RNAs (siRNAs) play central roles in controlling eukaryotic gene expression and have attracted increasing attention as potential therapeutic agents. In the cytoplasm, many of these RNAs are generated from cleavage of longer double-stranded RNA (dsRNA) substrates by the enzyme Dicer, a large multi-domain, Ribonuclease III-family protein 1; 2. However, the precursors to these RNAs are structurally distinct: pre-miRNAs are predicted to be imperfectly base-paired hairpins with a variable-size loop, whereas pre-siRNAs are usually perfectly base-paired duplexes of variable length with no loop. Some species have functionally distinct Dicers that process different kinds of substrates. For example, in *D. melanogaster* Dcr-1 and Dcr-2 are responsible for the biogenesis of miRNAs and siRNAs, respectively 3. However, there is only one known Dicer in the human system, which putatively processes both pre-miRNAs and pre-siRNAs. Understanding how processing rates for human Dicer are affected by

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substrate differences and by Dicer's protein binding partners is paramount to identifying the molecular mechanisms that underlie regulatory RNA production.

The catalytic core of Dicer, represented by the structure of the enzyme from *Giardia intestinalis*, comprises a PAZ domain N-terminal to two Ribonuclease III domains 4. This structure, likely to be common to all Dicer enzymes, led to a model for dsRNA recognition in which substrates are positioned for cleavage to produce dsRNA products of a specific 21–27 bp length 1. However, it remains unclear how Dicer accommodates substrate RNAs with the different helical structures and thermodynamic stabilities that characterize pre-miRNAs and pre-siRNAs.

In most eukaryotes, Dicer contains additional domains beyond those found in the *Giardia* enzyme, including an N-terminal DExH/D-box helicase, a domain of unknown function (DUF 283) and a C-terminal dsRNA binding domain (dsRBD) (Fig. 1A). These domains function at least in part to recruit dsRNA binding proteins that are central players in RNA-mediated gene silencing 5; 6; 7; 8.

Studies of RNA samples isolated from mammalian cells having or lacking Dicer show that a majority of Dicer products are miRNAs 9; 10. Some examples of endogenous siRNAs have been identified, but their overall occurrence is relatively uncommon in these cell types 11; 12. Nevertheless, siRNA precursors such as short hairpin RNAs (shRNAs) are thought to be processed by human Dicer, and some studies suggest that such processing has a vital role in enhancing siRNA efficiency by facilitating optimal RNA-induced silencing complex (RISC) loading 13; 14.

In addition to Dicer's potential for differential substrate recognition, double-stranded RNA binding proteins that directly interact with Dicer could influence its activity 15. These proteins, typically comprising 2–3 consecutive dsRBDs connected by variable-length linkers, include RDE-4 in *C. elegans* 16, R2D2 and Loquacious/R3D1 in *D. melanogaster* 17; 18 and TRBP and PACT in *H. sapiens* 5; 6. In *C. elegans*, RDE-4 is thought to be exclusively involved in the siRNA pathway 19. In *D. melanogaster*, the substrate preferences detected for R2D2 and Loquacious/R3D1 suggest roles in selecting pre-siRNA and pre-miRNA substrates, respectively, and in specifying guide strand selection during RISC assembly 18; 20; 21; 22. In humans, the HIV trans-activating response (TAR) RNA binding protein (TRBP) ensures efficient recruitment of miRNA onto Argonaute 2 (Ago-2) in RISC and enhances the stability and efficiency of both Dicer and RISC 5; 13; 23; 24; 25. Perhaps as a result, changes in steady-state levels of certain miRNA families correlate with TRBP truncation mutations that characterize some colorectal cancers 26. PACT has also been shown to bind to human Dicer 6; 25, though its effect on dsRNA binding or processing remains unclear.

To investigate the effects of substrate variants and TRBP on dicing kinetics, we compared the processing rates of two distinct dsRNA substrates containing two-nucleotide 3' overhangs: a 73-nucleotide hairpin with an imperfect stem (human pre-let-7a) and a 35-bp perfect duplex (Fig. 1A). Dicer showed a marked preference for processing pre-let-7a over the 35-bp duplex, an effect most pronounced under multiple turnover conditions in which substrate is in excess over enzyme. TRBP increased the rate of endonucleolytic cleavage of both substrates by ~4 to 5-fold under multiple turnover conditions. This stimulatory effect required the two N-terminal dsRBDs of TRBP. These results reveal substantial variability in Dicer-catalyzed small RNA processing depending on substrate sequence and secondary structure. Although TRBP stimulates dicing activity, substrate-dependent differences in dicing rates are inherent to human Dicer itself. Our findings imply that levels of miRNAs and siRNAs are influenced at least in part by precursor structure and/or sequence.

Furthermore, levels of regulatory RNA in the cell could change as a consequence of mutations that alter substrate RNA structure or stability.

Results and Discussion

Dicing rates vary with different substrates

In vitro, human Dicer will cleave various dsRNA substrates including pre-miRNAs and short or long perfect duplexes to generate miRNAs and siRNAs, respectively. These substrates are structurally distinct, leading to potential differences in dicing rates that could contribute to variability in miRNA and/or siRNA levels *in vivo*. To directly test dicing rates of these two substrate types, we designed a 73-nucleotide hairpin with the sequence of the human pre-miRNA pre-let-7a and a 35-bp perfect duplex representing a pre-siRNA. Both substrates contained 3' two-nucleotide overhangs for optimal recognition by Dicer (Fig. 1A).

We performed dicing assays under single turnover conditions, in which Dicer is present in large molar excess over the substrate concentration. Under these reaction conditions, the rate of product formation is not limited by product release since each enzyme reacts at most with one substrate molecule. Cleavage of pre-let-7a was considerably faster than that observed for the 35-bp duplex. The $t_{1/2}$ (time to cleave half of the starting material) for pre-let-7a was <5 minutes, whereas for the 35-bp duplex it was ~80 minutes (Fig. 1B).

Under multiple turnover conditions, in which substrate concentration is in molar excess over that of the enzyme, an even more striking difference in substrate processing rates was observed. With a 10-fold excess of substrate over Dicer, the $t_{1/2}$ for pre-let-7a was ~15 minutes, whereas <1% of the 35-bp duplex was cleaved in the same amount of time (Fig. 1C). Thus, Dicer catalyzes cleavage of multiple pre-let-7a substrates per enzyme molecule while being unable to process the 35-bp substrate in multiple turnover fashion under identical reaction conditions.

Equilibrium binding affinities of each substrate for Dicer are similar (Table 1), which suggests that the differences in rate and extent of Dicer-mediated cleavage with these substrates are not simply a consequence of substrate binding affinity. Other possible explanations include inefficient product release or sub-optimal substrate loading, resulting in catalytic defects and/or the formation of non-productive enzyme-substrate complexes. Electrophoretic mobility shift assays (EMSA) performed in prior studies, as well as our own filter binding data, showed that Dicer has a very low affinity for duplexes of length that represents dicing products (Table 1) 23. Additionally, the results from our single turnover experiments demonstrated that cleavage rates already differ by >10-fold under conditions in which product release should not influence the accumulation of product. We therefore speculate that the 35-bp duplex is "mis-loaded" onto Dicer, thereby retarding the progress of the reaction. This might not occur with pre-let-7a if its structural features preclude non-productive binding to Dicer. This possibility is consistent with the observation that *in vivo*, human Dicer is predominantly responsible for the production of miRNAs 9. It is noteworthy that even among pre-miRNAs, there naturally exist many variations in structure such as the size of the loop and the number of mismatches in the stem, which may lead to considerable differences in the way distinct substrates are recognized and processed by Dicer. Recent studies have revealed pre-miRNAs with structural properties that make them unsuitable substrates for Dicer and are instead processed by Ago-2 27; 28 It is therefore conceivable that Dicer has a specific set of substrate structural requirements that lead to considerable variability in catalytic efficacy.

TRBP has a stimulatory effect on dicing kinetics

TRBP binds and stabilizes Dicer, modifies Dicer's endonucleolytic activity and has been suggested to help load product dsRNAs onto Ago-2 during formation of RISCs 5; 23. In addition, TRBP truncations correlate with changes in the population distributions of certain endogenous miRNAs *in vivo* 26. Studies using chimeric constructs of RDE-4 and TRBP showed that the two N-terminal dsRBDs contribute largely to the dsRNA binding affinity of TRBP 29. Whether and how TRBP influences Dicer's selection and speed of dsRNA substrate processing has not been well characterized.

To test the influence of TRBP on dicing kinetics, recombinantly expressed Dicer and TRBP were incubated together to form a heterodimeric complex, which was then purified by size exclusion chromatography to remove any free TRBP 7. Control experiments showed that excess TRBP binds tightly to dsRNA and sequesters it from the Dicer-TRBP complex, inhibiting dicing without necessarily reflecting the behavior of the Dicer-TRBP complex itself (data not shown).

Equilibrium binding analysis of Dicer-dsRNA complexes in the presence and absence of TRBP, using an electrophoretic mobility shift assay, suggested that TRBP enhances complex stability with both pre-let-7a and the 35-bp duplex (Fig. 2A). The Dicer-TRBP complex gave rise to a slowly migrating ternary complex with either dsRNA substrate, whereas Dicer alone did not. To obtain quantitative information about dsRNA binding affinities, we performed filter binding assays to measure equilibrium dissociation constants (K_ds) of Dicer alone and the Dicer-TRBP complex bound to both substrates. Although there is little difference in binding affinities between the two substrates, the Dicer-TRBP-RNA complex has a significantly lower K_d compared to the Dicer-RNA complex without TRBP (<50 pM versus ~2 nM for pre-let-7a and <51 pM versus ~3.5 nM for the 35-bp duplex, Table 1).

We then performed dicing assays using the Dicer-TRBP complex. Under single turnover conditions, the purified Dicer-TRBP complex showed a slight stimulatory effect on dicing of the 35-bp duplex (Fig. 2B). While the $t_{1/2}$ for Dicer alone was ~80 minutes, it was ~45 minutes for Dicer-TRBP. In the case of pre-let-7a we found Dicer-TRBP to be identical to Dicer alone ($t_{1/2}$ of <5 minutes) (Fig. 2B). Under multiple turnover conditions, TRBP caused an increase in the rate and extent of dicing with both substrates. Notably, however, the presence of TRBP did nothing to ameliorate the relatively inefficient processing of the 35-bp duplex compared to pre-let-7a (Fig. 2C). With pre-let-7a, the $t_{1/2}$ for Dicer alone was ~15 minutes, compared to ~8 minutes for the Dicer-TRBP complex (Fig. 2C).

TRBP-mediated stimulation of dicing could occur at multiple stages of the dicing reaction trajectory. The much higher affinity of the Dicer-TRBP complex for RNA substrates, as compared to Dicer alone (Table 1), strongly suggests that TRBP accelerates dicing in part by assisting in substrate recruitment. It is also possible that substrate binding exerts some amount of conformational strain on Dicer, which TRBP may alleviate *via* its interaction with Dicer and by properly orienting the substrate in Dicer's active site. Furthermore, we cannot exclude the possibility that TRBP also plays a role facilitating product release and enzyme turnover. Future experiments will be needed to fully explain the mechanistic basis for TRBP's stimulatory effect on dicing.

TRBP requires high RNA binding affinity to stimulate dicing

In order to obtain a more detailed picture of the effect of substrate variation and the presence of TRBP on Dicer function, we performed dicing assays under multiple turnover conditions using a series of substrate concentrations. Michaelis-Menten plots obtained from these data revealed that Dicer's maximum cleavage rate (V_{max}) for pre-let-7a is 100-fold higher than

that for the 35-bp perfect duplex. We also find that the Dicer-TRBP complex shows a 4–5 fold increase in V_{max} over that measured for Dicer alone, irrespective of substrate type (Fig. 3A, B). Thus, the specific sequence and structure of RNA substrates have substantial effects on catalysis rates, resulting in V_{max} differences spanning multiple orders of magnitude. Furthermore, TRBP leads to an overall enhancement in cleavage rate, regardless of the specific substrate. Surprisingly, K_m values extracted from the Michaelis-Menten curves are in the tens of nanomolar range (Fig. 3A, B), both in the presence and absence of TRBP, whereas the K_ds for substrate binding are up to three orders of magnitude lower (Table 1). Because K_m does not equal K_d for these complexes, product formation may not be the rate-limiting step. For example, substrate molecules may need to undergo conformational changes after initial binding in order to form productive enzyme-substrate complexes.

The stimulation of dicing activity by TRBP could either be due to its dsRNA binding affinity or a conformational change brought about by protein-protein interactions between TRBP and Dicer, or a combination of both. Previous RNA-binding studies using wild-type or mutated forms of TRBP implicated the first two dsRBDs in RNA binding; while dsRBD2 was clearly shown to have the highest affinity for dsRNA 30, dsRBD1 also enhanced the RNA binding affinity of a TRBP-RDE4 chimera 29. In agreement, studies of truncation mutants showed that the C-terminal dsRBD3 is dispensable for RNA binding 29; 31 and contributes primarily to protein-protein interactions and Dicer binding 5; 32. Furthermore it has also been shown in *Drosophila* that a truncation mutant of Loquacious containing dsRBD2 and dsRBD3 is sufficient both for binding Dcr-1 (via dsRBD3) and enhancing Dcr-1 mediated miRNA production (via dsRBD2) 33.

In order to investigate the roles of the dsRNA-binding and Dicer-binding dsRBDs of TRBP in stimulating dicing, we designed a truncation construct ($TRBP_{RBD3}$) that lacked dsRBD1 and dsRBD2 but retained its ability to interact with Dicer. We were able to purify the Dicer-$TRBP_{RBD3}$ complex, which failed to produce an electrophoretic mobility shift with either the pre-let-7a or the 35-bp dsRNA substrate, similar to the behavior of Dicer alone (data not shown). Additionally, the measured binding affinity of Dicer-$TRBP_{RBD3}$ for either substrate is within error of the affinity measured for Dicer alone (Table 1). These observations strongly suggest that TRBP confers high dsRNA binding affinity to the Dicer-TRBP complex via its two N-terminal dsRBDs.

The Dicer-$TRBP_{RBD3}$ complex was tested in dicing assays with both pre-let-7a and the 35-bp duplex under multiple turnover conditions. In both cases, this enzyme complex produced little or no stimulation of dicing relative to the rate observed for Dicer alone (Fig. 3C). However, we have not completely eliminated the possibility that $TRBP_{RBD3}$ has a lower binding affinity for Dicer compared to that of full-length TRBP, and therefore dissociates appreciably during the reaction. Nevertheless, these data indicate that dsRBD2 – and perhaps dsRBD1 to a lesser extent – are required for TRBP's stimulatory affect on dicing. In agreement with studies of Loquacious and Dcr-1 from *Drosophila* 33, dsRBD3 is required for Dicer binding. Future studies of TRBP containing point mutations in dsRBD1 and dsRBD2 that inhibit RNA binding will be needed to investigate whether these domains are also involved in protein-protein interactions that affect dicing kinetics.

A TRBP phosphorylation mimic does not significantly affect dicing kinetics

The post-translational phosphorylation of TRBP *in vivo* has been shown to increase the apparent half-life of Dicer and affect cellular miRNA levels 24. TRBP expressed in *E. coli* is devoid of post-translational modifications, whereas TRBP expressed in a baculovirus system exhibits heterogeneous phosphorylation 24Enbo Ma and J.A.D., unpublished data). Therefore, to test whether the TRBP phosphorylation state affects Dicer's enzymatic activity, we purified a phospho-mimic mutant of TRBP ($TRBP_{pm}$) containing aspartate

residues at each of the four positions shown to be sites of phosphorylation *in vivo* (Fig. 1A). This protein was shown to recapitulate the effects of native phosphorylated TRBP in cultured human cells 24. We performed dicing assays using a Dicer-$TRBP_{pm}$ complex under multiple turnover conditions at substrate concentrations that were previously used for Michaelis-Menten analysis. We found that for both substrates used in this study, Dicer-$TRBP_{pm}$ – mediated cleavage did not differ significantly from that measured for Dicer-TRBP. While we see a 1.5 fold increase in the V_{max} for dicing the 35-bp duplex, the V_{max} for dicing pre-let-7a remained virtually identical (Fig. 3A, B).

Paroo *et al.* showed that the relative abundance of certain miRNAs varied between cells expressing the phospho-mimic TRBP mutant and those expressing a non-phosphorylatable TRBP mutant. Notably, in this study, let-7a was among the miRNAs whose abundance decreased in the $TRBP_{pm}$-containing cells. The absence of an effect of $TRBP_{pm}$ on dicing rates in this study suggests that instead of altering dicing kinetics, TRBP phosphorylation may influence miRNA levels by an indirect mechanism. A more detailed analysis with pre-miRNAs representative of both the up-regulated and down-regulated populations would nevertheless be desirable to evaluate the possibility that TRBP phosphorylation influences dicing kinetics in other contexts. We note that the dissociation constants for $TRBP_{pm}$:RNA complexes are comparable to those measured for wild-type TRBP (Table 1), consistent with the idea that TRBP stimulates dicing by a mechanism that generally depends on its RNA binding affinity.

Conclusions

Among Dicer-dependent small RNAs in mammalian cells, miRNAs are by far the most prevalent and well characterized 9; 10. The occurrence of endogenous siRNAs in mammalian cells is a recently discovered and relatively rare phenomenon 11; 12. It is therefore plausible that pre-miRNAs are the natural substrates for Dicer in human cells and that other kinds of small RNAs, if processed *in vivo*, are generated by Dicer-independent mechanisms. We propose that specific structural features of pre-miRNAs facilitate optimal loading and therefore efficient Dicer-mediated processing, which is precluded in the case of other substrates such as the pre-siRNA analyzed in this study (Fig. 4). The TRBP-dependent increase in dicing rates observed for both substrates, an effect that requires high-affinity RNA binding, suggests that TRBP assists in recruiting substrates to Dicer, as previously suggested in the *C. elegans* and *D.melanogaster* systems 29; 33; 34, and/or stabilizes the interaction between Dicer and its RNA substrates (Fig. 4). However, Dicer itself possesses an intrinsic ability to distinguish between substrates with different sequences and structural characteristics. Together, these results show that the specific nature of the dicing substrate heavily influences the catalytic production of regulatory RNAs and could therefore be an important aspect of small RNA biogenesis *in vivo* and for the development of RNAi-based therapeutics.

Materials and Methods

Sample preparation

Dicer, TRBP and Dicer-TRBP complexes were purified using published protocols 35. We used site-directed mutagenesis to incorporate the TRBP point mutants S142D, S152D, S283D and S286D, and purified $TRBP_{pm}$ 24 using the same procedure as for wild-type TRBP. Human pre-let-7a was synthesized by *in vitro* transcription using T7 RNA polymerase from a DNA template containing a double ribozyme system to ensure homogeneous 5′ and 3′ ends 36. The two strands of the 35-bp duplex (a and b) and those of the 19-bp duplex (a and b) were synthesized by IDT (Integrated DNA technologies, Coralville, IA). All substrates were purified using 16% urea-PAGE, and purified pre-let-7a,

35-bp duplex b and 19-bp duplex a were then 5' end-labeled using T4 polynucleotide kinase (New England Biolabs Inc., Beverly, MA) and (γ-^{32}P)-ATP. The substrates were heated at 65°C for 10 minutes in annealing buffer containing 3mM $MgCl_2$, 30mM NaCl, and 100mM Tris.HCl (pH 7.5), followed by flash cooling for refolding pre-let-7a and slow cooling to anneal the 35-bp and 19-bp duplexes. The sequences of the substrates are as follows:

pre-let-7a: 5′-UGAGGUAGUAGGUUGUAUAGUUUUAGGGUCACACCCACCACUGGGAGAUAACUAUACAAUCUACUGUCUUACC-3′

35-bp duplex a: 5'-UGAGGUAGUAGGUUGUAUAGUUUGAAAGUUCACGAUU-3'

35-bp duplex b: 5'-UCGUGAACUUUCAAACUAUACAACCUACUACCUCAAA-3'

19-bp duplex a: 5'-GUCACAUUGCCCAAGUCUCTT-3'

19-bp duplex b: 5'-GAGACUUGGGCAAUGUGACTT-3'

Kinetic assays

Single turnover assays were performed in 45 μL mixtures, which consisted of 25 nM enzyme and <1 nM 5' ^{32}P-labeled substrate. Upon incubation at 37 °C, 5 μL aliquots were removed and mixed with 6 μL of loading buffer at time points of 0.5, 1, 2.5, 5, 10, 20, 40 and 80 minutes. These samples were heated at 70 °C for 10 minutes prior to being run on a 16% urea-polyacrylamide gel and quantified with a phosphorimager (GE Healthcare). The data were plotted using Kaleidagraph (Synergy software). Multiple turnover dicing assays were also performed in 45 μL mixtures containing 5 nM enzyme and 5' ^{32}P-labeled substrate at concentrations of 25, 50, 75, 100, 150, 225 and 375 nM. Reactions were incubated at 37 °C and 5 μL aliquots were taken after 0.5, 1, 2.5, 5, 10, 20, 40 and 80 minutes. Variations in the time points were required in order to adapt to varying levels of dicing activity with different substrates and different proteins, as indicated in the figure axes. After quantification, initial velocities (v_o) at each substrate concentration (S) were determined by linear regression, and V_{max} along with standard errors were calculated by fitting to the Michaelis-Menten equation, $v_o = (V_{max} \times S)/(K_m + S)$, using Kaleidagraph.

Electrophoretic Mobility Shift Assay

35-bp duplex and pre-let-7a were annealed in 100 mM Tris-HCl (pH 7.5), 3 mM $MgCl_2$ and 30 mM NaCl by heating at 65°C for 10 minutes and either slow cooled (35-bp duplex) or flash cooled (pre-let-7a). ~10.5 picomoles of each of these RNAs were then incubated with equimolar Dicer, Dicer-TRBP or Dicer-$TRBP_{RBD3}$ for an hour at 4 °C in 20 mM Tris–HCl (pH 7.5), 25 mM NaCl, 5 mM EDTA, 1 mM DTT and 1% glycerol. Reactions were analyzed on a 6% non-denaturing polyacrylamide gel by staining with ethidium bromide.

Filter Binding Assays

Serial dilutions of Dicer, TRBP or Dicer-TRBP constructs were incubated with <1 nM of 5' ^{32}P-radiolabeled 35-bp duplex, pre-let-7a or 19-bp duplex in binding buffer containing 20 mM Tris-HCl (pH 7.5), 25 mM NaCl, 1 mM DTT, 1% glycerol and 0.01% Igepal CA-630. Reactions with Dicer were supplemented with 5 mM EDTA to prevent RNA cleavage by Mg^{2+} chelation. After incubating reactions (25 or 50 μL volume) on ice for one hour, samples were applied by vacuum to a dot-blot apparatus containing two pieces of Whatman filter paper below three membranes: 0.2 μm pore size Tuffryn (Pall Co.), 0.1 μm pore size Protran (Whatman) and Hybond-N (Amersham). The membranes were equilibrated in binding buffer for at least 20 minutes prior to use. After applying reactions and washing with 50 μL of binding buffer, membranes were dried and exposed overnight on a phosphor

screen. The amounts of free RNA (retained on Hybond-N membrane) and protein-bound RNA (retained on Protran membrane) were quantified using a phosphorimager (GE Healthcare). The extent of complex aggregation, as detected by radioactivity on the Tuffryn membrane, was negligible. The fraction of RNA bound, calculated as the ratio of radioactivity on the Protran membrane to the sum of radioactivity on the Protran and Hybond-N membranes, was plotted as a function of protein concentration. K_ds were determined by fitting the data to binding isotherms using KaleidaGraph (see Table 1 for details).

Research Highlights

- Human Dicer shows significant variability in dicing efficiency in a substrate sequence and/or structure dependent manner.
- We report a clear preference for pre-miRNAs over pre-siRNAs.
- The Dicer associated dsRNA binding protein TRBP engenders a ~ 5 – fold increase in endonucleolytic activity with both kinds of substrates.
- This stimulatory activity of TRBP is most likely dependent on the high RNA binding affinity attributable primarily to dsRBD2 and perhaps, to a smaller extent, also to dsRBD1.

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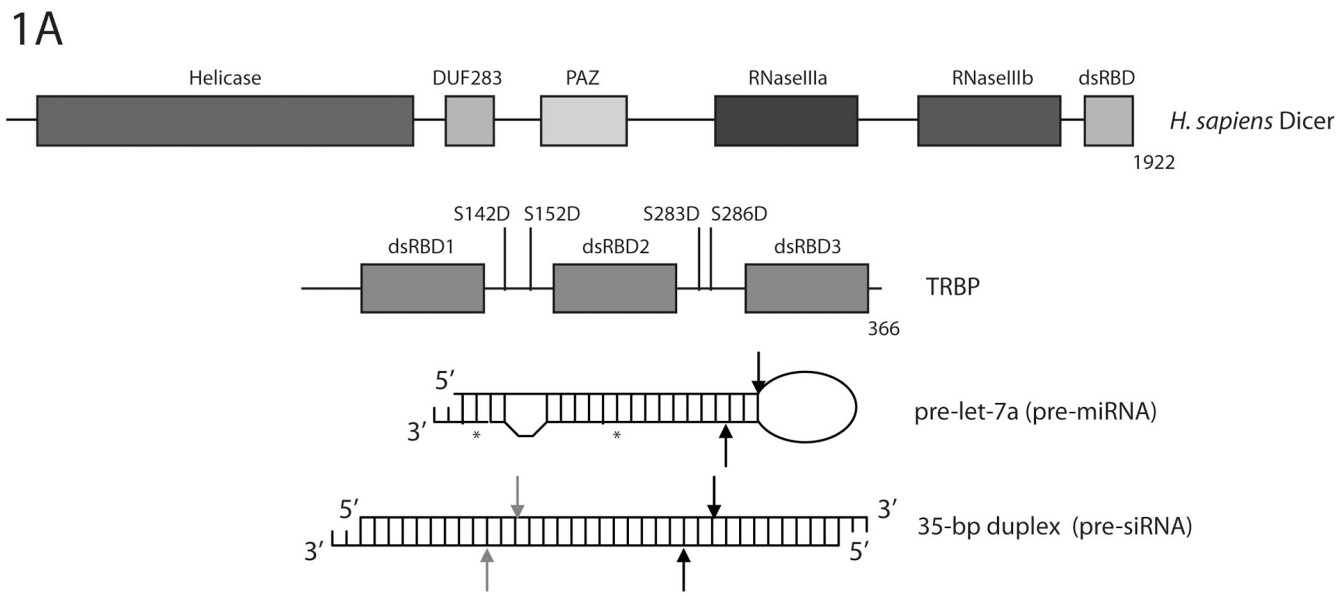
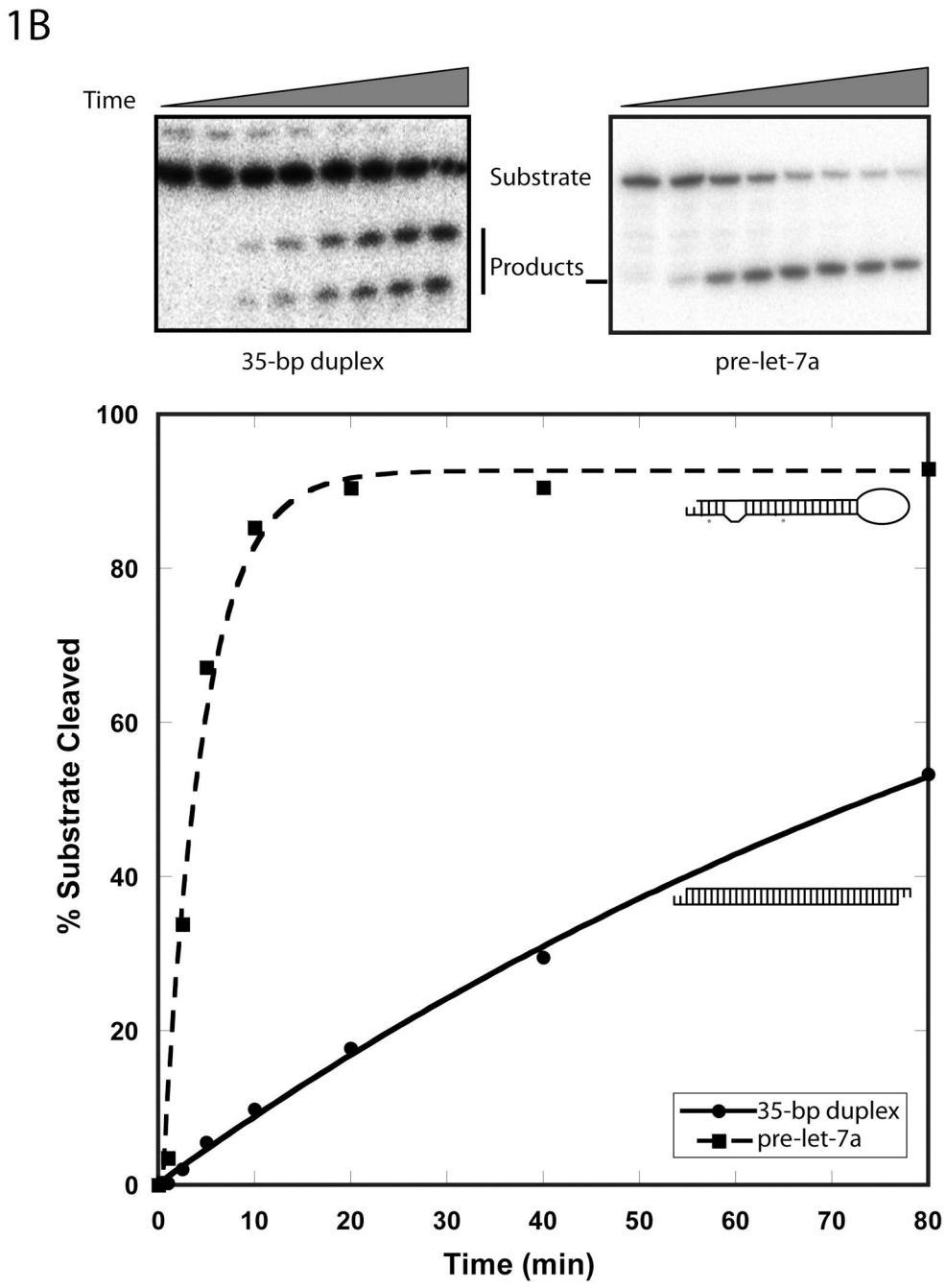




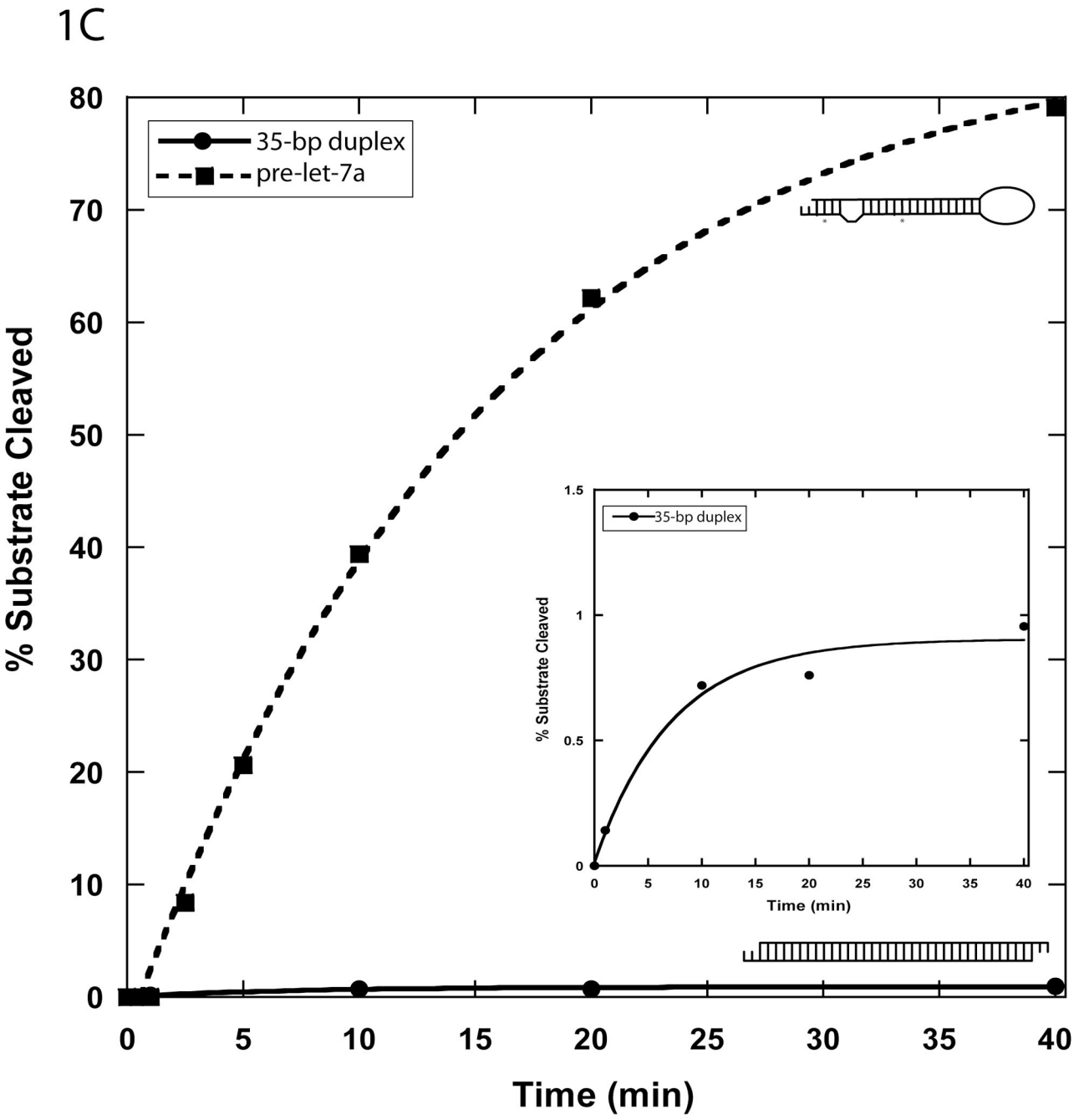
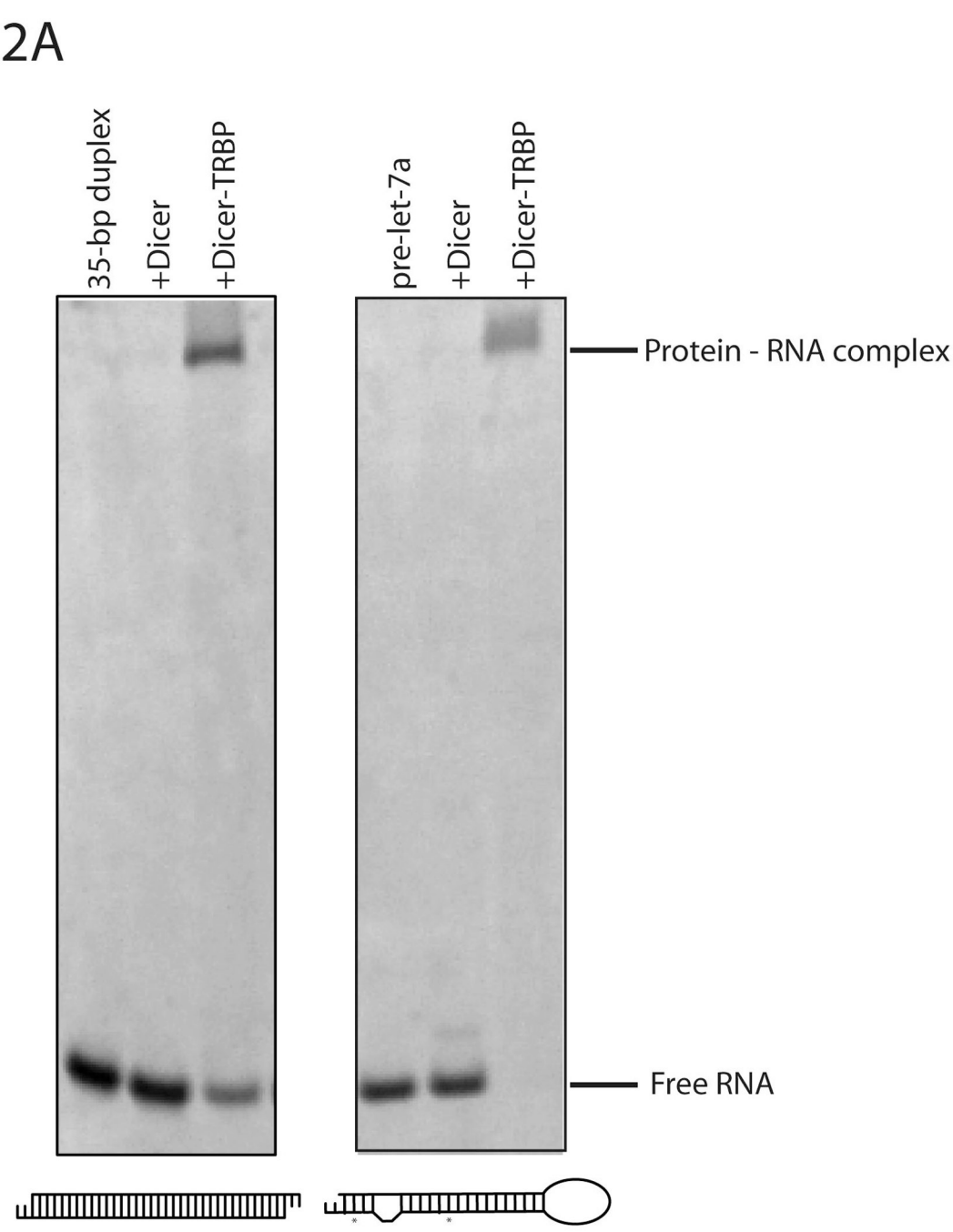


Figure 1.

(a) Schematic representation of the domain organization of the proteins involved in this study: full-length human dicer and TRBP. Mutations made to generate the phospho-mimic mutant of TRBP ($TRBP_{pm}$) are denoted. Also shown are cartoon representations of the two substrates used in this study, pre-let-7a and a 35-bp perfectly complementary duplex with 3' two-nucleotide overhangs. There are four G-U wobbles in the stem of pre-let-7a, two of which are predicted by Mfold 37 to be base-paired (depicted with asterisks) and two of which are predicted to be mis-matched (depicted with a bulge). Arrows indicate sites of Dicer-mediated cleavage. **(b)** Single turnover dicing assay with 35-bp duplex and human pre-let-7a using Dicer alone showing products of expected length based on a single pair of

cleavage sites for pre-let-7a and two pairs (one on either end) for the 35-bp duplex. Reactions contained <1 nM RNA and 25 nM Dicer, and were incubated at 37 °C. The 16% urea-polyacrylamide gels corresponding to the plots are shown above the graph. The plots for each substrate are marked by substrate icons, as depicted in (a). **(c)** Multiple turnover dicing assay with 50 nM 35-bp duplex and pre-let-7a using Dicer alone at a concentration of 5 nM. The plots for each substrate are marked by substrate icons, as depicted in (a). The inset shows an expanded y-axis of cleavage data for the 35-bp duplex.



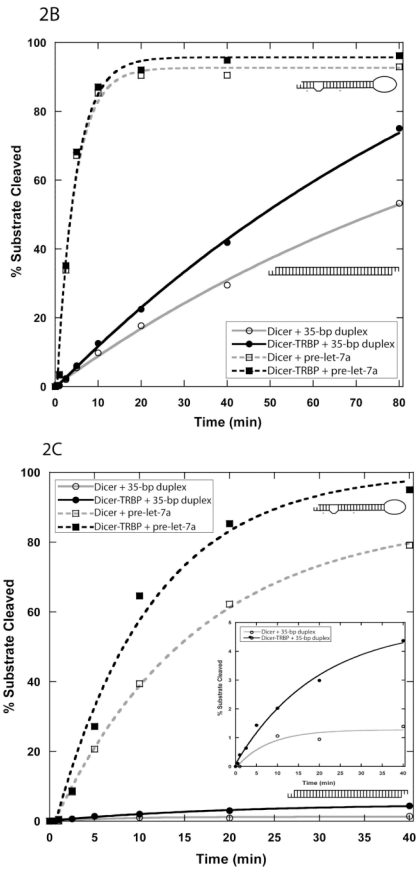
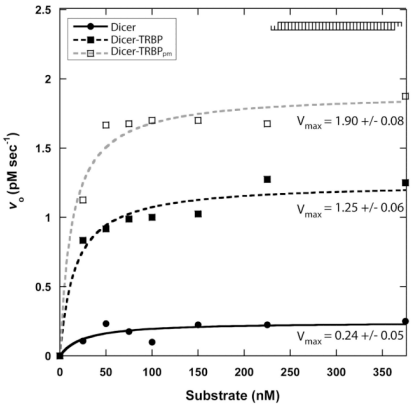
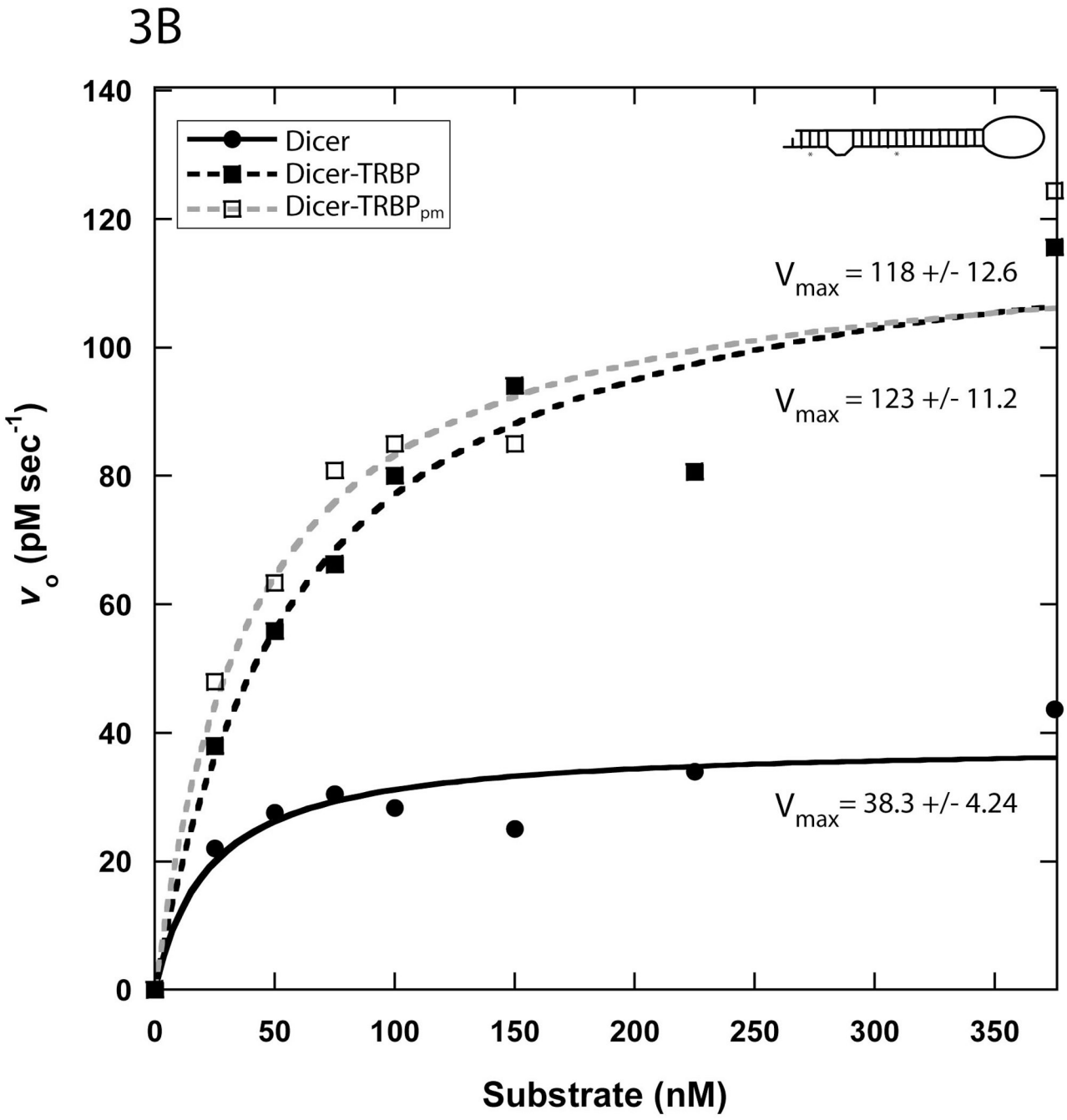


Figure 2.

(a) Electrophoretic mobility shift assay with both 35-bp duplex and pre-let-7a showing the relative tendencies of Dicer and Dicer-TRBP to form stable protein-RNA complexes. Binding reactions were analyzed with 6% non-denaturing PAGE and stained with ethidium bromide. The gels corresponding to each substrate are marked at the bottom by substrate icons, as depicted in 1a. **(b)** Single turnover dicing assay with 35-bp duplex and pre-let-7a using a Dicer-TRBP complex under conditions identical to 1b. The plots for Dicer alone are shown in grey for the purpose of comparison. The plots for each substrate are marked by substrate icons, as depicted in 1a. **(c)** Multiple turnover dicing assays with 25 nM 35-bp duplex and 50 nM pre-let-7a using a Dicer-TRBP complex under conditions identical to 1c. The plots for Dicer alone are shown in grey for the purpose of comparison. The inset shows an expanded y-axis of cleavage data for the 35-bp duplex.

3A





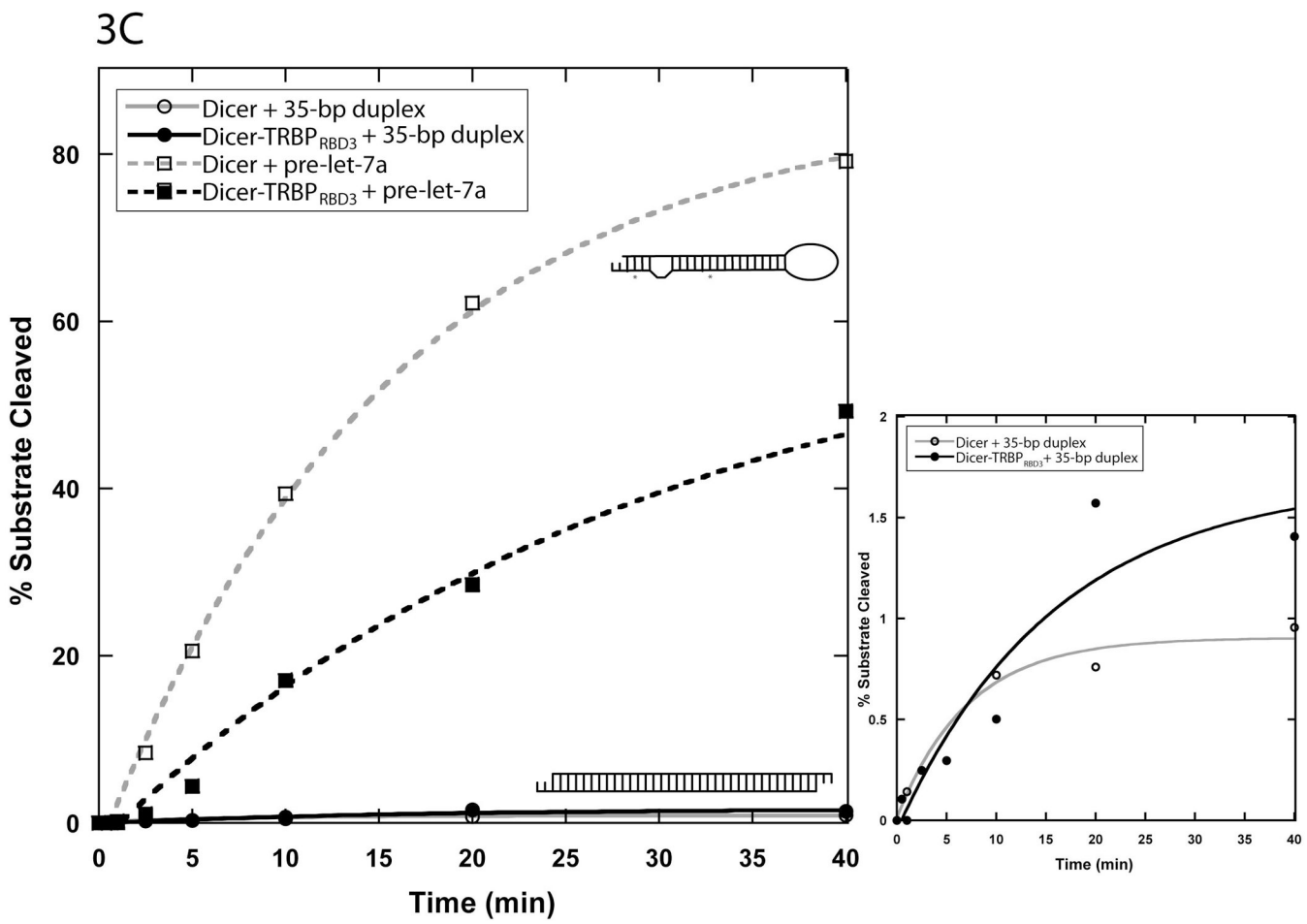


Figure 3.

(a) Michaelis-Menten analysis of dicing of the 35-bp duplex. 5 nM enzyme (Dicer, Dicer-TRBP or Dicer-TRBP$_{pm}$) was incubated with 5' ^{32}P-labeled substrate at the concentrations shown. **(b)** Michaelis-Menten analysis of dicing of pre-let-7a. 5 nM enzyme (Dicer, Dicer-TRBP or Dicer-TRBP$_{pm}$) was incubated with 5' ^{32}P labeled substrate at the concentrations shown. **(c)** Multiple turnover assays with the 35-bp duplex and pre-let-7a comparing the activity of Dicer-TRBP$_{RBD3}$ to that of Dicer alone. Cleavage data for the 35-bp duplex is re-plotted to the right with an expanded y-axis. The plots for each substrate are marked by substrate icons, as depicted in 1a.

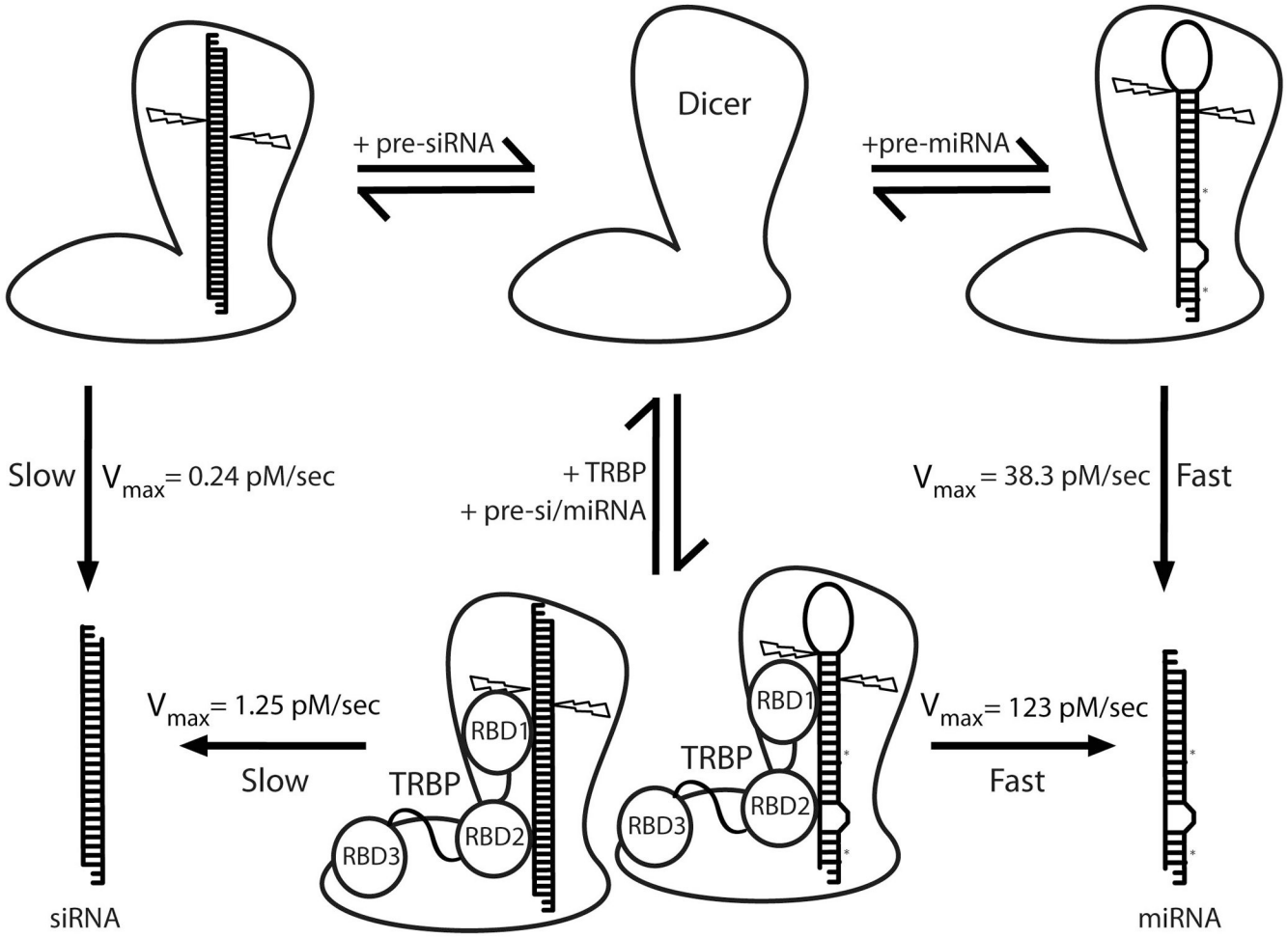


Figure 4.

Model depicting Dicer's substrate selectivity and the effect of TRBP on dicing kinetics. Cartoon representatives of Dicer and Dicer-TRBP are based on a low resolution structure determined by electron microscopy 8. The ~100 fold difference in V_{max} for pre-miRNA and pre-siRNA dicing could possibly reflect a difference in the loading efficacy, which in turn may be a function of the nature of the substrate. The N-terminal helicase domain of Dicer is known to bind the C-terminal dsRBD3 of TRBP, which in turn may mediate protein-nucleic acid interactions between the substrate RNA and one or both of the other dsRBDs of TRBP, thus stabilizing the enzyme-substrate complex. This could explain the 4–5 fold increase in activity of Dicer upon binding TRBP.

Table 1

Equilibrium dissociation constants (K_ds) for protein:RNA complexes

	Dicing Substrates		Dicing Product
	pre-let-7a	**35-bp duplex**	**19-bp duplex**
TRBP	1.3 ± 0.2 nM	< 90 pM[a]	0.29 ± 0.05 nM[b]
TRBP$_{pm}$	2.9 ± 0.6 nM	< 100 pM[a]	1.04 ± 0.07 nM[b]
Dicer	1.8 ± 0.4 nM	3.4 ± 0.5 nM	700 ± 100 nM[c]
Dicer-TRBP	< 50 pM[a]	< 51 pM[a]	0.24 ± 0.04 nM[b]
Dicer-TRBP$_{pm}$	< 40 pM[a]	< 52 pM[a]	ND
Dicer-TRB$_{PRBD3}$	1.3 ± 0.1 nM	3.0 ± 0.4 nM	ND

Equilibrium dissociation constants were determined using filter binding as described in the Methods. Data were analyzed using a standard binding isotherm unless otherwise stated, according to the equation: fraction bound = A × [protein] / (K_d + [protein]), where A is the amplitude of the binding curve. ND, not determined. Errors, where included, represent the standard fitting error.

[a] Because of the extremely low equilibrium dissociation constants for these complexes, it was not possible to lower the RNA concentration sufficiently below the K_d. Therefore, binding isotherms were fit with the solution of a quadratic equation describing a bimolecular dissociation reaction, as described previously[38]. K_ds are reported as an upper bound because the RNA concentration in the reactions could not be precisely determined, thereby limiting the accuracy of the linear regression fit.

[b] Binding curves displayed apparent negative cooperativity and were best fit by allowing a variable Hill coefficient (n = 0.4–0.6). Reported K_ds are $K_{0.5}$ values, i.e. the protein concentration at which the fraction RNA bound is equal to 50%.

[c] The low affinity of this complex prevented complete definition of the saturation regime in the binding curve. To avoid underestimating the K_d as a result, the amplitude was fixed (A = 1) when fitting the data. The reported Kd represents an upper bound estimate.