Human Dicer is an integral component of the RNA interference pathway. Dicer processes premicro-RNA and double-strand RNA to, respectively, mature micro-RNA and short interfering RNA (siRNA) and transfers the processed products to the RNA-induced silencing complex. To better understand the factors that are important for the binding, translocation, and selective recognition of the siRNA strands, we determined the binding affinities of human Dicer for processed products (siRNA) and short single-strand RNAs (ssRNA). siRNAs and ssRNAs competitively inhibited human Dicer activity, suggesting that they are interacting with the active site of the enzyme. The dissociation constants ($K_d$) for unmodified siRNAs were 5–11-fold weaker compared with a 27-nucleotide double-strand RNA substrate. Chemically modified siRNAs exhibited binding affinities for Dicer comparable with the substrate. 3’-Dinucleotide overhangs in the siRNA affected the binding affinity of human Dicer for the siRNA and biased strand loading into RNA-induced silencing complex. The $K_d$ values for the ssRNAs ranged from 3- to 40-fold weaker than the $K_d$ for the substrate. Sequence composition of the 3’-terminal nucleotides of the ssRNAs exhibited the greatest effect on Dicer binding. Dicer cleaved substrates containing short siRNA-like double-strand regions and extended 3’ or 5’ ssRNA overhangs in the adjacent ssRNA regions. Remarkably, cleavage sites were observed consistent with the enzyme entering the substrate from the extended 3’ ssRNA terminus. These data suggest that the siRNAs and ssRNAs interact predominantly with the PAZ domain of the enzyme. Finally, the tightest binding siRNAs were also more potent inhibitors of gene expression.

Post-transcriptional gene silencing by the mechanism known as RNA interference involves the processing of long double-strand RNAs (dsRNA)$^2$ into short effector dsRNAs by the RISC loading complex, Dicer (1, 2). Human cells contain a single Dicer isoform, which has been shown to generate both short interfering RNAs (siRNA) and mature micro-RNA products (3–6). siRNA and micro-RNA consist of sense and antisense strands relative to the target mRNA. The siRNA and microRNA products of Dicer are loaded into the RNA-induced silencing complex (RISC), resulting in either the translational repression or destruction of the mRNA target (7–9).

Dicer belongs to the RNase III family of enzymes and appears to be ubiquitous in eukaryotes. The enzyme is a 220-kDa protein consisting of a dsRNA-binding domain, a PAZ domain, two RNase III domains, and a domain homologous to ATP-dependent RNA helicase (10–13). The two RNase III domains of Dicer form an intramolecular dimer that together cleave the opposing strands of the dsRNA, generating 2-nucleotide-long 3’-overhangs (13). The crystal structure of Dicer from Giardia showed that the PAZ domain of the enzyme exhibited a tertiary structure similar to the PAZ domain of human Argonaute2 (14). In addition, the hydrophobic pocket of the PAZ domain was responsible for binding the 3’-dinucleotide overhang of the substrate (14). The PAZ and RNase III domains are separated by an $\alpha$-helix that functions as a molecular ruler to generate the ~20-base pair dsRNA products (14). Finally, the role of the ATPase/RNA helicase domain of the enzyme is unclear, since no helicase activity has been observed for the enzyme (10).

Dicer is a key component of the RISC loading complex (RLC) (2–5, 15). The minimum RLC consists of human Dicer and the human immunodeficiency virus transactivating response RNA-binding protein (TRBP) (16, 17). The role of the RLC in the activities of siRNAs is unclear. Several studies showed that the reduction of either human Dicer or TRBP protein expression reduced the activities of siRNAs (17, 18). Other studies showed that a reduction in Dicer protein expression appeared to have no effect on siRNA activity (19, 20). In addition, synthetic Dicer substrates ranging from 25 to 30 nucleotides were shown to be more potent inhibitors of gene expression than the corresponding 21-nucleotide siRNAs, further suggesting a role for human Dicer in the activities of siRNAs (21). In Droso phila, cross-linking studies between Dicer-2 and the TRBP paralog R2D2 bound to the siRNA indicated that Dicer-2 was positioned adjacent to the 5’ terminus of the sense strand, and R2D2 was positioned adjacent the 5’ terminus of the antisense strand (22). The directionality of the interaction appeared to be influenced by the base-pairing stabilities of the siRNA termini (22). Similarly, substrates containing 3’-overhangs were determined to affect the directionality of human Dicer cleavage as
well as the gene silencing activities of the siRNAs (23). Together, these data suggested that Dicer participates in the translocation of the cleavage product to RISC and that binding directionality between the enzyme and the product plays a role in the selective loading of the siRNA strands into RISC.

The substrate specificity of human Dicer has been characterized. The enzyme cleaves long double-strand RNA substrates from either terminus, producing cleavage products that are typically 21–25 base pairs long (10). Substrates containing 3’-overhangs exhibited faster cleavage rates compared with substrates with blunt termini, although both substrates exhibited similar binding affinities for the enzyme (24). The sequence composition of the 3’-overhangs as well as the length of the overhangs also affected the rate of cleavage, with no cleavage observed for overhangs longer than 3 ribonucleotides (24).

It is surprising, given that the enzyme is believed to participate in the translocation of the products to RISC that much less is known about the interaction between human Dicer and siRNA products it produces. Provost et al. (25) showed by electrophoretic mobility shift assay Dicer did not bind siRNA, suggesting that the enzyme may not be directly involved in the translocation of the products of Dicer cleavage (siRNAs) into RISC. Thus, there is a need to resolve the issue of whether Dicer indeed translocates siRNA to RISC and how this is accomplished. To better understand the interaction between human Dicer and its siRNA cleavage products as well as the properties important for the translocation of the siRNA from Dicer to RISC, we determined the binding affinities of human Dicer for substrate and various native and chemically modified siRNAs and short single-strand RNAs (ssRNA). Based on the observed binding interactions between the enzyme and the siRNAs and ssRNAs, substrates containing short siRNA-like double-strand regions and extended 3’ or 5’ single-strand RNA regions were prepared, and the Dicer cleavage activities were determined. Finally, we compared the gene silencing activities of various modified and unmodified siRNAs in cultured cells with the binding affinities of human Dicer for the siRNAs.

**EXPERIMENTAL PROCEDURES**

**Preparation of Oligonucleotides and 32P-Labeled Substrate—** Synthetic oligoribonucleotides were manufactured by Dharmacon Research, Inc. siRNA duplexes were formed according to the manufacturer’s instructions. The double (siRNA) and single strand (ssRNA) structures of the oligoribonucleotides were confirmed by native polyacrylamide gel electrophoresis. The alternating 2’-fluoro-, 2’-methoxy-modified oligonucleotides were prepared as previously described (26). The final concentration of the duplex was 20 μM in 100 mM potassium acetate, 2 mM magnesium acetate, 30 mM HEPES-KOH, pH 7.4. The 27-base pair RNA substrate was 5’-end-labeled with [γ-32P]ATP, T4 polynucleotide kinase, and standard procedures (27). The labeled substrate was purified on a 12% polyacrylamide gel. The specific activity of the labeled oligonucleotide was ~3000–8000 cpm/fmol.

**Preparation of Recombinant Human Dicer—** The human Dicer cDNA clone was purchased from OriGene Technologies, Inc. (Rockville, MD). The GST and His tags were engineered into the N and C termini of the cDNA of the pcDNA3.1/V5-HIS plasmid (OriGene Technologies). The GST-Dicer-His cDNA was then subcloned into the pENTR 2B plasmid (Invitrogen). The plasmid was recombined with the BaculoDirect Linear DNA to create the Baculo Virus expressing GST- and His-tagged human Dicer in sf9 cells. The virus was amplified and transfected into sf9 cells. The cells were harvested 3 days following transfection, and lysate was subjected to two affinity purifications. First, His tag affinity purification with the Talon column (Clontech) and elution of the enzyme with 80 mM imidazole and 80 mM EDTA were performed. Second, the eluant was GST affinity-purified with the GST column according to the manufacturer’s instructions (Amersham Biosciences). The purity of the Dicer protein was more than 95%.

**Multiple-turnover Kinetics—** The 27-base pair RNA substrate ranging in concentration from 100 nM to 10 μM was digested with 200 ng of human Dicer in cleavage buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2.5 mM MgCl2) at 37 °C. At times of 2–120 min, a 10-μl aliquot of the cleavage reaction was quenched with 5 μl of stop solution (8 M urea and 120 mM EDTA). Cleavage reactions were analyzed by denaturing polyacrylamide gel electrophoresis as described and quantitated on an Amersham Biosciences PhosphorImager. The concentration of the converted product was plotted as a function of time. The initial cleavage rate was obtained from the slope (mol of RNA cleaved/min) of the best fit line for the linear portion of the plot, which comprises in general <10% of the total reaction and data from at least five time points. Alternatively, cleavage products were analyzed by ion pair HPLC electrospray mass spectrometry. Specifically, samples were phenol/chloroform-extracted and injected directly on a Waters XBridge column (Waters, Milford, MA). Separation was accomplished using an Agilent 1100 HPLC-mass spectrometry system (Agilent, Wilmington, DE). The column was maintained at 55 °C, and flow rate on the column was 0.25 ml/min. The column was equilibrated with 35% acetonitrile in 5 mM tributylammonium acetate, pH 7.0. A gradient from 35 to 80% acetonitrile over 15 min was used to elute the RNA duplex and dicer processing products, and the single strands were detected in the mass spectrometer. Mass spectra were obtained using a spray voltage of 4 kV, a sheath gas flow of 35 p.s.i.g., a drying gas flow rate of 12 liters/min at 325 °C, and a capillary voltage of −125 V. Under these conditions, oligonucleotides ranging in length from 15 to 27 nucleotides are detected in the −4 and −5 charge state. Chromatograms and mass spectra were analyzed using Agilent Chemstation software. Each peak was manually averaged for its m/z value, and the results were compared with a table containing the calculated m/z values of expected Dicer products.

**Determination of Dissociation Constants (K_d)—** Binding affinities were determined by inhibition analysis. Here, the initial cleavage rates were determined for the 27-base pair RNA substrate at a variety of concentrations in both the presence and absence of a competing noncleavable substrate analog (i.e. siRNA, ssRNA, and dsDNA), as described for multiple-turnover kinetics. The dsRNA substrate was prepared as described above except in 50 μl of cleavage buffer with final concentrations ranging from 200 to 800 nM. The competing noncleavable substrate analog was prepared in 50 μl of hybridization buffer ranging in concentration from 1 to 10 μM. The reac-
Human Dicer Binds Short Single-strand and Double-strand RNA

siRNA Treatment—HeLa cells were cultured in DMEM supplemented with 10% fetal calf serum, streptomycin (0.1 μg/ml), and penicillin (100 units/ml). Treatment of cells with siRNA was performed using Opti-MEM medium (Invitrogen) containing 2–5 μg/ml Lipofectamine 2000 (Invitrogen) for 3–5 h at 37 °C, as described previously. For the generation of IC_{50} curves, cells were seeded in 96-well plates at 4000–6000 cells/well and then treated at doses ranging from 2 pM to 60 nM in half-log serial dilutions. Treated cells were incubated overnight. The next day, total RNA was purified from 96-well plates using an RNeasy 3000 BioRobot (Qiagen, Valencia, CA). Reduction of target mRNA expression was determined by quantitative RT-PCR. mRNA levels were normalized to total RNA for each sample as measured by Ribogreen (Invitrogen). IC_{50} curves and values were generated using Prism 4 software (GraphPad).

Immunoprecipitation of Overexpressed Ago2 for the RISC Activity Assay—The cDNA of human Ago2 with an N-terminal HA epitope was subcloned into the mammalian expression vector phCMV-2. HeLa cells (CCL-2) were treated with the Ago2 expression plasmid using Effectene transfection reagent. 24 h later, cells were treated with 75 nM synthetic oligoribonucleotides or siRNAs using Lipofectamine 2000 reagent and incubated for an additional 18 h. Cells were harvested with trypsin and washed twice with 1 ml of cold phosphate-buffered saline. The cell pellet was resuspended in 500 μl of lysis buffer (150 mM NaCl, 0.5% Nonidet P-40, 2 mM MgCl_{2}, 2 mM CaCl_{2}, 20 mM Tris at pH 7.5, protease inhibitor, and 1 mM dithiothreitol) and passed through an insulin syringe. Supernatants were clarified with a 10,000 g clear spin for 10 min, and protein concentrations were determined. 1.8 mg of total protein was incubated with 15 μl of HA II beads (Covance) for 2 h, washed three times with lysis buffer, and equilibrated in cleavage buffer (10 mM Tris at pH 7.5, 100 mM KCl, 2 mM MgCl_{2}, protease inhibitor, 0.5 mM dithiothreitol). 0.1 mM ^{32}P-labeled target RNA was added, and cleavage reactions quenched at the specified

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**FIGURE 1.** Ion pair HPLC electrospray mass spectrometry analysis of 27-base pair substrate digested with human Dicer. A, mass spectra of the 27-base pair substrate incubated with (top and center) and without (bottom) human Dicer. The charge states for the products are shown above the corresponding peak. B, sequences of the detected products are provided along with the strand lengths (n), the calculated molecular weights, and observed molecular weights based on the observed charge states from A. C, sequence of sense (top) and antisense (bottom) strands of the 27-base pair substrate are shown, respectively, 5'→3' and 3'→5'. The arrows indicate the positions of the Dicer cleavage sites corresponding to the most abundant cleavage products detected by liquid chromatography-mass spectrometry.
**RESULTS**

siRNA and ssRNA Are Competitive Inhibitors of Dicer Substrate—$K_i$ values for substrate, the cleavage products of Dicer (siRNA), and short ssRNA were determined by inhibition analysis. We chose inhibition analysis for several reasons. First, this method allows the measurement of a wide range of dissociation constants, including weaker binding affinities (i.e., those in the high nanomolar to micromolar range) (28). Second, this method provides information about the nature of the enzyme/ligand interaction. Specifically, the type of inhibition, (e.g., competitive, uncompetitive, noncompetitive) gives an indication of whether the inhibitor is binding to the reactive site on the enzyme, modifying the substrate, or causing an allosteric effect on the enzyme. Lineweaver-Burk and Augustinsson analysis of the data were used to determine the inhibitory constant ($K_i$), and the $K_i$ is equivalent to the $K_d$ of the enzyme when a noncleavable inhibitor is used (28).

The enzyme cleaved the 27-base pair substrate, generating 21–25-base pair products (Figs. 1 and 2A). To determine the binding affinity of human Dicer for substrate, a 27-base pair noncleavable substrate was prepared with 2’-methoxyribonucleotide substitutions at the cleavage sites (Fig. 2A). No human Dicer cleavage activity was observed for the noncleavable 27-base pair duplex containing 2’-methoxyribonucleotide substitutions, the 19-base pair siRNA (si92), or the 19-nucleotide single-strand RNA (s92) (Fig. 2A). Lineweaver-Burk analysis of the initial cleavage rates for the 27-base pair RNA substrate in the presence and absence of the si92 siRNA or s92 single-strand RNA demonstrated that si92 and s92 were competitive inhibitors of the 27-base pair substrate (Fig. 2, B and C). The noncleavable 27-base pair duplex containing 2’-methoxy ribonucleotide substitutions at the cleavage sites also functioned as competitive inhibitor of the 27-base pair substrate (data not shown). The $K_d$ values for the noncleavable 27-base pair duplex, si92, and s92 were 47, 451, and 154 nM, respectively (Fig. 2A).

**Human Dicer Binds siRNA Products and Short Single-strand RNAs**—Previous studies have shown that the thermodynamic stabilities of the terminal base pairs of the siRNA duplex as well as 3’-overhangs appear to influence the binding directionality of the siRNA to the RLC (22). To evaluate the effects of thermodynamic stabilities of the terminal base pairs on the interaction with the enzyme, we designed 19-base pair siRNAs containing terminal base pairs predicted to exhibit similar (si92) and asymmetrical (si97) thermodynamic stabilities and siRNAs with various types of overhangs (Table 1).

The dissociation constants for the 19-base pair si92 and si97 duplexes were $\sim$7–12-fold weaker compared with the $K_d$ for the 27-base pair noncleavable substrate (Table 1A and Fig. 2A). Dicer exhibited a slightly tighter binding affinity for the si97 duplex with G:C and A:U base pairs at the termini, compared with the si92 duplex with A:U terminal base pairs (Table 1A).

**Human Dicer Binds Short Single-strand and Double-strand RNA**

![Image](49x364 to 407x733)

**FIGURE 2.** siRNA and single-strand RNA are competitive inhibitors of human Dicer substrate. A, denaturing polyacrylamide gel analysis of substrates incubated without human Dicer (lanes 1, 3, 5, and 7) or with human Dicer (lanes 2, 4, 6, and 8). The arrows indicate the bands corresponding to the Dicer cleavage products. The underlined sequences represent 2’-methoxy substitutions. The arrows indicate positions of Dicer cleavage. Dissociation constants ($K_i$) were determined by competitive inhibition. The positions of the $^{32}$P label (p) are shown in boldface type. Dicer digestion of 27-base pair substrate (lanes 1 and 2), 27-base pair noncleavable substrate (lanes 3 and 4), 19-base pair siRNA (si92) (lanes 5 and 6), and 19-nucleotide single-strand RNA (s92) (lanes 7 and 8). B and C, Lineweaver-Burk analysis of human Dicer cleavage activities of the 27-nucleotide RNA duplex alone (triangle) and in the presence of 300 nM si92 single strand RNA (box) or 900 nM si92 siRNA (circle). Initial cleavage rates ($V_i$) were determined as described under “Experimental Procedures.”

Times were measured in gel loading buffer (Ambion). Cleavage reactions were analyzed by denaturing polyacrylamide gel electrophoresis and quantitated with an Amersham Biosciences PhosphorImager.
The 5′-phosphate appeared to have no effect on Dicer binding, since comparable dissociation constants were also observed for the si92 duplexes containing 5′-phosphates (si92/s5′-PO, si92/as5′-PO, and si92/sas5′-PO) and the si92 siRNA (Table 1A). Similarly, comparable binding affinities were observed for the si97 duplex containing a 5′-phosphate (si97/as5′-PO) and the si97 siRNA (Table 1A). Finally, human Dicer exhibited a significantly weaker binding affinity for the double-strand deoxyribonucleotide duplex (si92/DNA) compared with si92.

To explore the effects of the dinucleotide 3′-overhang on Dicer binding, a series of duplexes was prepared in which purine and pyrimidine ribonucleotide and deoxyribonucleotide overhangs were positioned at either the 3′ or 5′ terminus of the sense and antisense strands of the si92 and si97 duplexes (Table 1B). These duplexes containing 5′-dinucleotide overhangs exhibited \( K_d \) values comparable with the length-matched si92 duplex, suggesting that the 5′-overhangs have no effect on the binding affinity of human Dicer for the siRNA (Table 1B).
Human Dicer Binds Short Single-strand and Double-strand RNA

position of the 3’-overhang (e.g. 3’ terminus of the sense strand versus the antisense strand) of the siRNA appeared to have a greater influence on human Dicer binding compared with the nucleotide composition of the 3’-overhang. Specifically, human Dicer exhibited a 2–7-fold tighter binding affinity for the si92 duplexes containing the 3’-diribonucleotide overhang in the antisense strand compared with the si92 duplexes with the 3’-overhang in the sense strand (Table 1B). In contrast, varying the dinucleotide composition in the 3’-overhang of the antisense strand resulted in only a 2-fold difference in binding affinity. Similarly, a maximum 2-fold difference in binding affinity was observed for the various nucleotide combinations at the 3’-terminus of the sense strand. (Table 1B). Finally, the influence of the 3’-overhang on human Dicer binding appears to be independent of duplex sequence. For example, a tighter binding affinity was also observed for the si97 duplex containing the thymidine deoxyribonucleotide overhang at the 3’ terminus of the antisense strand (si97/as3’-dTdT) compared with the duplex overhang at the 3’-terminus of the sense strand (si97/s3’-dTdT) (Table 1B). Taken together, these data suggest that although the thermodynamic stability of the terminal base pairs plays a role, the dinucleotide 3’-overhangs exhibit a greater effect on human Dicer binding.

Table 2 shows the binding affinities of human Dicer for single-strand RNAs. The binding affinities for the various single-strand oligonucleotides differed significantly compared with the siRNA duplexes. Specifically, human Dicer exhibited an ~14-fold tighter binding affinity for the sense strand (s92) compared with the antisense strand (as92) of the si92 duplex (Table 2A). The tightest binding affinities were observed for the single-strand RNAs that contained purine dinucleotides at the 3’ terminus compared with single-strand RNAs with 3’-terminal pyrimidine dinucleotides. Remarkably, the binding affinity for s92 was only 3-fold weaker than the binding affinity for the non-cleaveable 27-base pair substrate (Table 2A). A similar trend was observed for the single-strand RNAs of the si97 duplex in which Dicer bound the sense strand tighter than the antisense strand, although a smaller 2’-fold difference in binding affinity was observed (Table 2B). The 2’-fluoro/2’-methoxy substitutions reversed the rank order for binding. Specifically, Dicer exhibited a 2-fold tighter binding affinity for the modified antisense strands (as92/2’-F/OMe and as97/2’-F/OMe) compared with the modified sense strands (s92/2’-F/OMe and s97/2’-F/OMe) (Table 2A). Finally, significantly weaker binding affinities were observed for the single-strand RNAs containing thymidine deoxynucleotides at the 3’ terminus of the sense and antisense strands (Table 2A and B).

To better understand the observed differences in the human Dicer binding affinities for single-strand sense and antisense RNAs, s92 and as92 were designed with additional uridine residues positioned at both termini of s92, and adenine residues were positioned at both termini of as92 (Table 2C). The dinucleotide substitution at the 3’ terminus of the oligoribonucleotide significantly affected human Dicer binding affinity. Again, Dicer exhibited the tightest binding affinities for the single-strand RNAs that contained purine dinucleotides compared with pyrimidine dinucleotides at the 3’ terminus. For example, human Dicer exhibited 8–12-fold tighter binding affinities for the oligoribonucleotides containing adenine dinucleotides at the 3’ terminal compared with the oligoribonucleotides containing 3’-uridine dinucleotides (Table 2C). In contrast, dinucleotide substitutions at the 5’ terminal appeared to have no effect on Dicer binding, since comparable binding affinities were observed for s92 and the s92/5’-UU and as92/5’-AA duplexes (Table 2C). In addition, these data suggest that the influence of the dinucleotide substitutions on Dicer binding affinity is not due to the increased length of the ssRNA.

Human Dicer Cleaves Substrates Containing Extended Single-strand Overhangs from the 3’ Single-strand Terminus and within the 3’ and 5’ Single-strand Regions—Given the observed strong binding affinity of human Dicer for siRNAs and single-strand RNAs, we asked whether human Dicer could bind and cleave substrates containing short siRNA-like double-strand regions. We prepared a series of substrates containing double-strand RNA regions ranging from 5 to 21 nucleotides (Table 2). In addition, substrates containing flanking 3’- or 5’ single-strand RNA regions ranging from 5 to 13 nucleotides exhibited 8–12-fold tighter binding affinities for the unmodified RNA duplexes compared with the siRNA duplexes (Table 2). The 2’-fluoro/2’-methoxy substitutions reversed the rank order for binding. Specifically, Dicer exhibited a 2-fold tighter binding affinity for the modified antisense strands (as92/2’-F/OMe and as97/2’-F/OMe) compared with the modified sense strands (s92/2’-F/OMe and s97/2’-F/OMe) (Table 2). Finally, significantly weaker binding affinities were observed for the single-strand RNAs containing thymidine deoxynucleotides at the 3’ terminus of the sense and antisense strands (Table 2).
Human Dicer binds short single-strand and double-strand RNA

Human Dicer cleavage pattern for length-matched 25-base pair duplexes. Shown is denaturing polyacrylamide gel analysis of substrates incubated with (+) and without (−) human Dicer. Reactions were incubated at 37 °C for 90 min. Substrate structures are shown below the gels with the nucleotide positions on the sense strand (top sequence) numbered from 5′ to 3′ and antisense strand (bottom sequence) numbered from 3′ to 5′. Substrates were prepared with either a 32P-labeled sense strand (gels 1 and 3) or a 32P-labeled antisense strand (gels 2 and 4). The positions of the 32P label (p) are shown in boldface type. The arrows indicate the positions of the Dicer cleavage sites for each substrate. Each 32P-labeled strand was digested with RNase T1 (T1) to determine the positions of the Dicer cleavage sites.

FIGURE 3. Human Dicer cleavage pattern for length-matched 25-base pair duplexes. Shown is denaturing polyacrylamide gel analysis of substrates incubated with (+) and without (−) human Dicer. Reactions were incubated at 37 °C for 90 min. Substrate structures are shown below the gels with the nucleotide positions on the sense strand (top sequence) numbered from 5′ to 3′ and antisense strand (bottom sequence) numbered from 3′ to 5′. Substrates were prepared with either a 32P-labeled sense strand (gels 1 and 3) or a 32P-labeled antisense strand (gels 2 and 4). The positions of the 32P label (p) are shown in boldface type. The arrows indicate the positions of the Dicer cleavage sites for each substrate. Each 32P-labeled strand was digested with RNase T1 (T1) to determine the positions of the Dicer cleavage sites.

otides in length were tested. Each substrate contained double-strand blunt (B), double-strand overhang (O), and single-strand (S) termini (Figs. 4 and 5). Given that human Dicer cleaves the substrate at a fixed distance from either end of the duplex, we used the position of Dicer cleavage to determine the binding directionality of the enzyme on the substrates (24). To evaluate the Dicer cleavage activities at both termini, the substrates were prepared with the 32P label at either the 5′ terminus of the sense strand, 3′ terminus of the sense strand, or 5′ terminus of the antisense strand. Finally, the Dicer cleavage of these substrates was compared with cleavage of 25-base pair length-matched substrates of the same sequence (Fig. 3).

Human Dicer cleavage of the 25-base pair length-matched substrates resulted in the predicted Dicer cleavage products ranging from 19 to 23 base pairs (Fig. 3) (24). The cleavage sites at positions 3–5 and 21–23 indicated that the enzyme cleaved the substrates from both blunt termini. The extent of the cleavage activity appeared to be similar for both 3′-sense/5′-antisense and 5′-sense/3′-antisense termini cleavage products (Fig. 3).

The same Dicer cleavage sites at positions 3–5 and 21–25 were observed for the substrates containing 23- and 25-base pair double-strand regions and 3′ single-strand overhangs in the sense strand (23/3′-7a and 25/3′-5a and -b) (Figs. 3 and 4, A and B). The positions of these cleavage sites were consistent with Dicer entering the substrates from both the blunt end (B) and overhang termini (O) of the double-strand regions (Fig. 4, A and B). Interestingly, new cleavage sites were observed at positions 7 and 10 in the sense strand of the 23/3′-7a and 25/3′-5a substrates and 18 and 19 in the antisense strand of the 25/3′-5a substrate that were not observed in the 25-base pair length-matched substrate (Figs. 3 and 4, A and B). The new cleavage products appear to be too short to be generated by Dicer entering the substrates from either the blunt end or overhang termini of the duplex. Instead, the new cleavage sites are 20–23 nucleotides from the 3′ single-strand RNA terminus of the substrate, suggesting that the cleavage sites were generated from the 3′ single-strand RNA terminus (5′ cleavage products) (Fig. 4, A and B). Similar cleavage sites at positions 5–10 were observed for the substrates containing double-strand regions 21 base pairs or shorter (21/3′-9a, 19/3′-11a, 17/3′-13a, 17/3′-13b, and 15/3′-15) and, given the shorter length of the double-strand regions of these substrates, strongly suggests that these cleavage sites are generated from the 3′ single-strand termini (S) and not the blunt end (B) or overhang (O) termini (Fig. 4, A and B). Finally, thymidine deoxyribonucleotide overhangs at the 3′ terminus of the antisense strand appeared to have no effect on the positions of Dicer cleavage (17/3′-13b and 17/3′-15) (Fig. 4, A and B).

To evaluate the Dicer cleavage activities closest to the 3′ single-strand RNA overhang, the substrates were labeled at the 3′ terminus of the sense strand with 32P-labeled cytidine bisphosphate (3′,32PCp) (Fig. 4, C and D). Once again, cleavage sites at positions 22–25 were observed for the 3′,32PCp labeled substrates (25/3′-5c and 23/3′-7b) (Fig. 3). The 3′,32PCp-labeled substrates containing 17–21-base pair double-strand regions (21/3′-9b, 19/3′-11b, 17/3′-13c, and 17/3′-13d) exhibited a single cleavage site at position 22 (Fig. 4, C and D). Remarkably, the cleavage site for these substrates was positioned in the single-strand RNA overhang region. In contrast to the 5′,32PCp-labeled substrates, no cleavage sites at positions 7 and 10 were observed for the 3′,32PCp-labeled substrates (Fig. 4). The lack of Dicer cleavage at these positions suggested that the 3′,32PCp was interfering with the enzyme activity at these sites. To test this hypothesis, unlabeled cytidine bisphosphate (pCp) was ligated to the 3′ terminus of the sense strands of the 5′,32PCp-labeled 25/3′-5 and 19/3′-11 substrates (25/3′-5d and 19/3′-11c) (Fig. 4, C and D). Again, no cleavage sites at positions 7 and 10 were
Human Dicer Binds Short Single-strand and Double-strand RNA

observed at positions 6, 7, and 10 of the 5′ structures of the 3′ sense strand (Fig. 4, C and D). Similarly, no Dicer cleavage sites were observed at positions 6, 7, and 10 of the 5′, 32P-labeled 19/3′-11c substrate with 3′-pCp. The addition of a cytidine residue at the 3′ terminus of the sense strand of the 25/3′-5a and 19/3′-11a substrates did not inhibit Dicer cleavage at positions 7–10 (data not shown). Taken together, these data suggest that the 3′-phosphate in the sense strand of the substrate is interfering with the interaction between the enzyme and 3′ single-strand RNA region of the substrate.

Fig. 5 shows the Dicer cleavage activity for the substrates containing double-strand RNA regions with flanking 5′ single-strand RNA regions. Similar cleavage patterns were observed for the 25-base pair length matched substrate and the substrates containing a 25-base pair double-strand region with a flanking 5′ single-strand region (25/5′-5a, -b, and -c) (Figs. 3 and 5). The cleavage sites were consistent with the enzyme entering the substrate from the blunt end (B) and overhang terminus (O) of the substrates (Fig. 4). Importantly, no Dicer cleavage sites generated from the flanking 5′ single-strand RNA regions of the 25/5′-5 substrates were observed (Fig. 5). A single cleavage site at position 7 was observed for the 17–21-base pair substrates (17/5′-13, 19/5′-11, and 21/5′-9) (Fig. 5). Once again, the cleavage sites were positioned within the adjacent single-strand RNA region (Fig. 5).

3′-Overhangs in siRNA Influence RISC Loading—Dicer exhibited a tighter binding affinity for the si92 siRNA containing the 3′-overhang in the antisense strand (si92/as3′-dTdT) compared with the duplex with the 3′-overhang in the sense strand (si92/si3′-dTdT) (Table 1B). To determine whether the binding affinities of Dicer for these siRNAs influence the selective loading of the siRNA strands into RISC, the si92/as3′-dTdT, si92/s3′-dTdT, and length-matched si92 siRNAs were transfected into HeLa cells expressing HA-tagged human Argonaute2 enzyme (HA-Ago2). HA-Ago2 was then immunoprecipitated from the transfected cells and incubated with a 40-nucleotide 32P-labeled sense RNA, and the cleavage activity of HA-Ago2 was used to estimate the relative amounts of the siRNA strands loaded into Ago2 (Fig. 6). Given that the 32P-labeled sense RNA is complementary to the antisense strand of the siRNA, if the antisense strand of the siRNA is favorably loaded into Ago2 compared with the sense strand, the Ago2 cleavage activity should be enhanced. Alternatively, if the sense strand of the siRNA is favorably loaded into Ago2, the cleavage activity should be reduced.
The immunoprecipitated Ago2 enzyme from the cells treated with the various siRNAs cleaved the target RNA in an Ago2-dependent manner (i.e. a single cleavage site between the 10th and 11th nucleotide from the 5' terminus of the antisense strand was observed in the target RNA) (Fig. 6, A and B). The cleavage activity observed for si92/as3'-dTdT, which exhibited the tightest binding affinity for human Dicer, was more than 2-fold greater compared with the cleavage activities observed for si92 and si92/s3'-dTdT (Fig. 6C). In other words, 100% more antisense strand than sense strand appeared to be loaded into the enzyme (Fig. 6C). In addition, these data suggest that the 3'-thymidine deoxyribo- nuclease overhang biases Ago2 loading in favor of the strand with the thymidine deoxyribonuclease overhang.

**Chemical Modification of the siRNA Enhanced Dicer Binding and Gene Silencing Activity in Cell Culture**—Dicer binding affinities and gene silencing activities of the unmodified and chemically modified si92 and si97 siRNAs are shown in Table 3. The gene silencing activities of the siRNAs were determined by measuring the reduction in PTEN target mRNA from HeLa cells transfected with various concentrations of the siRNAs. The 50% inhibitory concentration (IC50) for the various siRNAs were calculated from the dose-response curves.

The dissociation constants for the si92 duplexes containing alternating 2'-fluoro-, 2'-methoxy-modified ribonucleotides (si92/2'-F/OMe and si92/2'-F/OMe:as5'-PO) were ~2-fold tighter than the Kd observed for unmodified si92 (Table 3A). The modified duplexes also showed a similar improvement in potency with an ~2–3-fold lower IC50 observed for si92/2'-F/OMe and si92/2'-F/OMe:as5'-PO compared with unmodified si92 and si92/as5'-PO (Table 3A). A similar trend was observed for the duplexes containing 3'-overhangs. The si92/as3'-dTdT duplex exhibited a tighter binding affinity and greater potency compared with the si92/s3'-dTdT and length-matched si92 duplexes (Table 3A). The modified si92 duplexes also exhibited tighter binding affinities and greater gene silencing activities (Table 3B). In this case, the modified si97 duplexes exhibited a significantly greater enhancement in the gene silencing activities compared with the observed enhancement in binding affinities (Table 3B). For example, the modified si97 duplexes exhibited 5–7-fold tighter binding affinities and 20–30-fold lower IC50 values compared with the unmodified si97 duplexes (Table 3B). Taken together, these data suggest that the effects of modified nucleotide substitutions on both Dicer binding and siRNA potency may be influenced by the sequence of the duplex and that the binding affinity of Dicer for siRNAs affects potency but that the correlation is not precise, suggesting that other factors may be involved in the greater potency of the modified si97 siRNAs.
DISCUSSION

Human Dicer Avidly Binds siRNA Product and Short Single-strand RNAs—We determined the binding affinity of Dicer for two different siRNA sequences, ranging in length from 19 to 21 base pairs and consisting of length-matched siRNAs, siRNAs with 3' and 5' -dinucleotide overhangs, and chemically modified siRNAs. The siRNAs and ssRNAs functioned as competitive inhibitors of substrate, suggesting that these nucleic acid structures bind to the active site of the enzyme (Fig. 2). The range of binding affinities observed for the various siRNAs included $K_d$ values that were comparable to substrate to 20-fold weaker than substrate (Tables 1 and 3). The ssRNAs exhibited binding affinities ranging from 3- to 50-fold lower than the binding affinity for substrate (Table 2). Our results differ from a previous report that showed that Dicer did not bind siRNAs using an electrophoretic mobility shift assay (25). At least two factors may explain the differences between our study and that of Provost et al. (25). Compared with the electrophoretic mobility shift assay, the competitive inhibition assay used here has been shown to be useful in measuring a wider range of dissociation constants, particularly weaker binding affinities (e.g. $K_d$ values in the high nanomolar to low micromolar range) (28). A second factor is that in this study, we addressed this question more intensively with multiple siRNAs. Importantly, the $K_d$ of 47 nM observed for the noncleavable 27-base pair substrate by competitive inhibition is consistent with the 50–60 nM $K_d$ values observed for longer substrates determined using either nitrocellulose filter binding or an electrophoretic mobility shift assay (24, 25).

The predominant factor influencing the binding affinity of human Dicer for siRNA was the position of the 3' -overhang in the siRNA (Table 1). The composition of the 3' -overhang appeared to play a secondary role with respect to Dicer binding. For example, the Dicer binding affinities for the si92 siRNA sequences varied as much as 7-fold, exhibiting a rank order for Dicer binding to the si92 siRNA as follows: 3' -overhang in the antisense strand, length-matched duplex, 3' -overhang in both the sense and antisense strand, 3' -overhang in the sense strand (Table 1). Within the siRNA groups containing the 3' -overhangs in either the sense or antisense strands, the nucleotide composition within the overhang accounted for a smaller, 2-fold, variance in binding affinity. A similar trend was observed for the si97 siRNA sequences (Table 1). The influence of the 3' -overhang on Dicer binding is probably due to the PAZ domain of Dicer, which has been shown to

TABLE 3
Comparison of Dicer binding affinity with potency in cultured cells for siRNA constructs

$K_d$ and 50% inhibition constants ($IC_{50}$) were determined as described under “Experimental Procedures.” $K_d$ and $\Delta K_d$ are reported as described in Table 1. siRNA constructs contained ribonucleotides (black), 3' -phosphate (PO), 2' -fluoro (green), and 2' -methoxy (blue) modified nucleotides.

A, si92 siRNA constructs. B, si97 siRNA constructs.
interact with 3′-overhang structures and direct the positioning of the enzyme on the substrate (14, 24). The tighter binding affinities observed for the siRNAs containing 3′-overhangs in the antisense strand compared with the sense strand suggest that the preferred binding directionality for these duplexes is with the PAZ domain of the enzyme positioned at the 3′ terminus of the antisense strand (Fig. 7A). In the case of the siRNAs containing 3′-overhangs at both the sense and antisense strands and the length-matched siRNAs for which no preferential binding directionality is predicted, the observed binding affinities are probably the sum of the interactions with both termini (Fig. 7A). In fact, these siRNAs exhibited $K_d$ values that were intermediate between those observed for siRNAs with 3′-overhangs in either the sense or antisense strands (Table 1). Because differences in binding directionality result in Dicer binding to different sequences within the siRNA, our data suggest that the interaction of Dicer with the siRNA is sensitive to base composition. In fact, the basic amino acid residues of the substrate-binding surface of Dicer are predicted to form extensive electrostatic interactions with the phosphates of the substrate, and differences in the helical conformation would be predicted to have an affect on these interactions (14). Consistent with this observation, Dicer bound the B-form DNA duplex more weakly than the A-form RNA duplex (Table 1A). Interestingly, we did not see a difference in Dicer binding affinities for the siRNAs containing 3′-DNA overhangs compared with siRNAs with 3′-RNA overhangs, again suggesting that the base composition within the duplex region of the siRNA plays a greater role in Dicer binding than the composition of the 3′-overhang (Table 1 (bottom)). Finally, these results differ from previous reports that showed that siRNAs with 3′-RNA overhangs were more potent than siRNAs with 3′-DNA overhangs, suggesting that additional factors may be contributing to the activities of siRNA molecules (24, 29, 30).

Very different results were observed for the ssRNAs. In this case, the binding affinity of Dicer for the ssRNAs appeared to be influenced solely by the 3′-dinucleotide composition. Dicer bound the ssRNAs containing purine dinucleotides at the 3′ terminus on average 15-fold tighter compared with the ssRNAs containing 3′-pyrimidine nucleotides. In contrast, the nucleotide composition at the 5′ terminus or 5′-phosphate substitutions had no effect on Dicer binding (Table 2). The strong influence of the 3′-terminal nucleotide composition on the binding affinity of Dicer for the ssRNAs suggests that the ssRNAs predominantly bind via the PAZ domain of the enzyme (Fig. 7B). The enhanced Dicer binding affinities for ssRNAs with 3′-terminal purine dinucleotides is consistent with the co-crystal structure of the PAZ domain from Argonaute2 bound to RNA, showing that the aromatic amino acids in the hydrophobic binding pocket of PAZ form stacking interactions with the two 3′-terminal nucleotides, although these result differ from molecular modeling studies, which suggested that uridine dinucleotides would form the most stable complex with the PAZ domain (31–33). The 2′-fluoro and 2′-methoxy substitutions in the ssRNAs reversed the rank order for binding, suggesting that 2′-sugar substitutions also affect PAZ binding (Table 2). Conversely, deoxyribonucleotide substitutions appear to have no effect on Dicer binding (Table 2).

**Dicer Cleavage Activity Is Consistent with the Observed Binding Affinities for siRNAs and ssRNAs**—We show that human Dicer binds siRNA and ssRNA with high affinity, suggesting that it is possible for Dicer to cleave substrates containing siRNA or ssRNA structures. We prepared substrates containing short siRNA-like duplex regions with flanking single-strand RNA regions (Figs. 4 and 5). These substrates contained three different termini: double-strand blunt (B), double-strand overhang (O), and single-strand (S) (Figs. 4, 5, and 8). Finally, we used the position of cleavage to determine the binding directionality of Dicer on the substrate (24).

The cleavage pattern observed for the substrates containing 3′ single-strand overhangs in the sense strand was consistent with the enzyme binding to the double-strand blunt (B), double-strand overhang (O), and single-strand (S) termini of the substrate (Figs. 4 and 8B). In the case of the substrates containing a 3′ single-strand overhang in the sense strand and double-strand regions shorter than the minimum length of ssRNAs, the cleavages occurred within the single-strand overhang region immediately outside the double-strand region (Figs. 4, C and D, and 8B). Interestingly, a pCp substitution at the terminus of the 3′ single-strand overhang ablated the cleavage sites generated from the 3′ single-strand terminus (S), suggesting that the 3′-pCp substitution interferes with Dicer binding (Figs. 4, C and D, and 8B). The effect of the pCp substitution on Dicer activity is consistent with the reported structure of PAZ, which predicts that the 3′-phosphate would sterically interfere with the nucleic acid binding pocket of PAZ (31).

In contrast to the substrates containing a 3′ single-strand overhang in the sense strand, cleavage patterns for the substrates containing 5′ single-strand overhang regions suggest that the cleavage sites were generated only from either the double-strand blunt (B) or overhang (O) termini (Figs. 5 and 8C). No cleavage sites generated from the 5′ single-strand termini...
addition, Dicer is capable of binding to 3′ single-strand regions but not the 5′ single-strand and cleaving within the adjacent double-strand region. Thus, Dicer is promiscuous in both binding and cleavage behavior, permitting it to participate in cleavage and RISC loading but also making it vulnerable to nonspecific cleavage activities and to competitive inhibition by a wide range of nucleic acid structures.

**Dicer Binding Specificity Translates to siRNA Activity**—The observed binding affinities of Dicer for its cleavage products (i.e. siRNAs) and ssRNAs are consistent with the enzyme’s role as a member of the RLC family in which Dicer transfers its cleavage products to RISC. Exactly how the translocation of the siRNA from the RLC to RISC is accomplished is unclear, but at some point during this process, the double-strand siRNA product of Dicer must be converted to a single-strand agent (i.e. antisense strand once loaded into Ago2 must hybridize to the target mRNA). It is unclear whether the conversion of the double-strand RNA to the single-strand species occurs before, during, or after the translocation from the RLC to RISC.

Our results provide a mechanism for the reported effects of siRNA structure on the interaction of the siRNA with the RLC and the selective loading of the siRNA strands (S) were observed (Figs. 5 and 8C). Again, for the substrates containing double-strand regions shorter than the minimum length of siRNAs, the cleavages occurred within the single-strand overhang region immediately outside the double-strand region (Figs. 5 and 8C). The ability of a double-strand-specific RNase to cleave adjacent single-strand RNA regions was also reported for *Escherichia coli* RNase H1 (34). Cleavage occurred exclusively within the 3′-single-strand RNA overhang, and cleavage sites were observed up to 3 nucleotides from the end of the DNA/RNA heteroduplex. In contrast, human Dicer cleaved both the 3′- and 5′-overhangs at distances up to 7 nucleotides from the duplex (Figs. 3 and 4).

Taken together, these data are consistent with the observed binding specificity of human Dicer. Specifically, Dicer is capable of binding to siRNA regions within a substrate, but given that the double-strand region is shorter than the minimum length for cleavage, the cleavage occurs within the single-strand overhang region immediately adjacent the siRNA region. In addition, Dicer is capable of binding to 3′ single-strand regions but not the 5′ single-strand and cleaving within the adjacent double-strand region. Thus, Dicer is promiscuous in both binding and cleavage behavior, permitting it to participate in cleavage and RISC loading but also making it vulnerable to nonspecific cleavage activities and to competitive inhibition by a wide range of nucleic acid structures.

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(W)
appears to be due to both the preferential loading of the antisense strand into Ago2 and the enhanced binding affinity of Dicer for the siRNA, resulting in more siRNA loading into RISC. Based on our model in which Dicer binding affinity for siRNAs and ssRNAs is influenced predominantly by the PAZ interaction with the 3′ terminus of the strands of the duplex, the predicted binding orientation for preferentially loading the antisense strand of the siRNA into RISC is with the PAZ domain of Dicer positioned at the 3′ terminus of the antisense strand (Fig. 7A). In the absence of 3′-overhang structures, the thermodynamic stability of the ends of the length-matched siRNA duplex would have a greater influence on this interaction, given that the PAZ interaction requires a free 3′ terminus. Consequently, the less stable A:U base pairs would be favored over G:C base pairs. In addition, the binding affinities observed for ssRNA suggest that Dicer would preferentially bind the strand containing purine compared with pyrimidine residues at the 3′ terminus. In fact, the HA-Ago2 cleavage activity for the length-matched si92 duplex suggests that the sense strand containing adenine residues at the 3′ terminus was preferentially loaded into Ago2 over the antisense strand containing uridine residues at the 3′ terminus (Fig. 6).

The alternating 2′-fluoro/2′-methoxy-modified siRNAs were previously shown to be more potent inhibitors of gene expression compared with unmodified siRNAs (26). Consistent with those observations, chemical modification of the siRNAs with alternating 2′-fluoro/2′-methoxy substitutions enhanced the potency of the si92 and si97 siRNAs (Table 3). In addition, Dicer exhibited tighter binding affinities for the modified siRNAs compared with the unmodified siRNAs (Table 3). Again, the enhancement in potency may be due to both tighter binding affinity for Dicer and enhanced bias toward antisense strand loading. Specifically, the rank order for binding to modified ssRNAs was reversed, with tighter binding affinities observed for the antisense strands of the si92 and si97 duplexes with the sense strands, which would bias the binding directionality of Dicer to preferentially load the antisense strands of the modified siRNAs into RISC (Table 3 and Fig. 7A). In the case of the si92 duplexes, the enhancement in binding affinity appeared to correlate with the enhancement in the potency of the modified siRNAs compared with the unmodified duplexes (Table 3 (top)). The modified si97 duplexes, on the other hand, showed a significantly greater (20–30-fold) enhancement in potency compared with the 5–7-fold enhancement in binding affinity over the unmodified siRNAs (Table 3 (bottom)). Given that the maximal enhancement in siRNA activity due to preferential strand loading would be 2-fold, these two factors alone cannot account for the significantly greater improvement in potency observed for the modified si97 duplexes. Clearly, other factors are contributing to the activities of the siRNAs.

In conclusion, our results demonstrate that Dicer can bind to a wide range of oligoribonucleotides and that the affinities observed are consistent with Dicer participating in both the cleavage of the siRNA precursors and the translocation of the siRNA products to RISC. We demonstrate that the 3′ terminus is crucial in binding interaction of the enzyme with the substrate and that this process is sensitive to base composition. This may provide an explanation as to why in only some cases Dicer substrates have been shown to be more potent than the comparable siRNAs. Moreover, Dicer appears to be promiscuous in both binding and cleavage, since the enzyme is capable of cleaving in double-strand and single-strand regions and can also bind to single-strand regions and cleave in a duplex. Clearly, Dicer interacts with other proteins, such as TRBP and Ago2 (17). Future studies should address how these protein-protein interactions affect the binding and cleavage properties of Dicer.

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