

Modified dsRNAs that are not processed by Dicer maintain potency and are incorporated into the RISC

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Received September 22, 2009; Revised January 18, 2010; Accepted January 19, 2010

ABSTRACT

Chemical modification of RNA duplexes can provide practical advantages for RNA interference (RNAi) triggering molecules including increased stability, safety and specificity. The impact of nucleotide modifications on Dicer processing, RISC loading and RNAi-mediated mRNA cleavage was investigated with duplexes ≥ 25 bp in length. It is known that dsRNAs ≥ 25 bp are processed by Dicer to create classic 19-bp siRNAs with 3'-end overhangs. We demonstrate that the presence of minimal modification configurations on longer RNA duplexes can block Dicer processing and result in the loading of the full-length guide strand into RISC with resultant mRNA cleavage at a defined site. These longer, modified duplexes can be highly potent gene silencers, with EC50s in the picomolar concentration range, demonstrating that Dicer processing is not required for incorporation into RISC or potent target silencing.

INTRODUCTION

RNA interference (RNAi) is a cellular process by which different classes of small RNAs regulate gene expression, silence transposon relocation and coordinate diverse cellular pathways (1). The first demonstrations of RNAi in mammalian cells utilized 19-bp duplexes with 2-nt 3'-end overhangs ('classical' siRNA) that were meant to mimic the natural products of Dicer cleavage of longer RNA duplexes (2). Two major classes of RNA duplexes are in use for achieving RNAi-mediated silencing for research and therapeutic applications: 'classical' 19-bp siRNAs, and longer duplexes typically in the range of 25–27-bp (3,4). Chemical modification of both types of RNA duplex is important for many aspects of therapeutic RNA compound optimization including the prevention of

off-target effects, reduction of immune stimulation and increased serum stability (5–8). While there have been both *in vitro* and cellular mechanism of action studies examining classical siRNAs (9), little has been reported for chemically modified duplexes with both approaches.

Some of the current models of RNAi action with exogenous double-stranded (ds) RNA duplexes include assumptions with regard to duplex length, requirement for activity of the RNase III-class endonuclease Dicer, and the involvement of accessory proteins in loading the duplex into the RNA induced silencing complex (RISC). In fact, Dicer substrate RNA duplexes have been reported to have dramatically increased gene silencing activity (4). However, it is unclear whether this can be attributed to the sequence of the processed duplex or a characteristic of the original length. Furthermore, a role has been proposed for Dicer in increasing the efficiency of incorporation of RNA duplexes into the RISC by association with the Argonaute (Ago) proteins (4,5). In mammalian cells, only the Ago2 protein has slicer activity resulting in cleavage and elimination of mRNA transcripts (10). Other Ago family members repress translation of message and may be responsible for activities characteristic of microRNA (miRNA) mediated gene silencing (10). While Ago2 is required for endonucleolytic RISC mediated gene silencing in mammalian cells, it is less clear if Dicer plays a necessary role in this process (11). For example, cells that lack the Dicer enzyme still support efficient gene silencing by RNAi at levels equal to that of Dicer-expressing cells (12).

To explore the mechanism of action of chemically modified duplexes in the RNAi pathway, we studied several 25–27-bp dsRNAs with potent silencing activity. Several aspects of the RNAi mechanism were examined using a chemical modification pattern that did not decrease silencing ability. We show that the guide strands of these longer duplexes are able to load into the RNAi machinery without being processed by the Dicer enzyme. Our results illustrate that not only is Dicer

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processing not necessary for longer duplexes to be efficient at silencing, but also that a classical siRNA configuration is not an absolute requirement for RISC loading and RNAi.

MATERIALS AND METHODS

Chemically synthesized RNAs

Single-stranded (ss) RNA was synthesized by Integrated DNA Technologies (Coralville, Iowa) or Thermo Scientific Dharmacon RNAi technologies (Lafayette, Colorado). RNA duplexes were formed by mixing equal molar ratios of the passenger and guide strands and incubating at 90°C for 1 min followed by 37°C for 1 h (2). All ssRNA and RNA duplexes were stored at -20°C. See Supplementary Tables S1 and S2 for RNA sequence information.

Dose response transfections

HEK293 cells (ATCC, Manassas, Virginia) were cultured in Dulbecco's Modified Eagle Media (DMEM) with 10% Fetal Bovine Serum and 1% Penicillin–Streptomycin. NIH3T3 Cells (ATCC, Manassas, Virginia) were cultured in DMEM with 10% Bovine Calf Serum and 1% Penicillin–Streptomycin. All cells were incubated at 37°C with 10% CO₂ as recommended by ATCC. Cultured cells were reverse-transfected with 25 nM total RNA duplex containing 0.005–5 nM active RNA duplex using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, California) transfection reagent as described by the manufacturer. Transfection complexes were prepared by adjusting the final concentration of total duplex to 25 nM with a non-targeting control duplex. The non-targeting chemistry and length matched control duplex targets the luciferase gene. Transfections were performed in 96-well plates with media containing no antibiotics and were incubated for 48 h under normal growth conditions. Cells were lysed and target mRNA (SOD1) was measured using the QuantiGene bDNA hybridization assay and gene specific probes (Panomics, Fremont, California) using assay conditions described by the manufacturer. The PPIB gene was used as a housekeeping control to normalize the SOD1 expression since it is not affected by SOD1 silencing. The percent silencing and EC50 values were based on comparing the normalized values of the samples transfected with SOD1 dsRNA to that of the normalized expression of SOD1 for the non-targeting control duplex. For duplexes targeting the PPIB gene, calculations were carried out similarly to SOD1 targeting duplexes by using the SOD1 gene to normalize expression. The values for EC50 analysis were calculated and graphically displayed using KaleidaGraph (Synergy Software, Reading, Pennsylvania).

Argonaute-2 immunoprecipitation and extraction of complexed RNA from RISC

RNA duplexes were annealed as described and transfected into 293T cells stably expressing c-myc Ago2 (Hannon Lab, Cold Spring Harbor Labs, Cold Spring Harbor,

New York) (9). 293T cells were cultured as described above in the presence of 0.5 µg/ml G418 to selectively express c-myc Ago2. Transfection of RNA duplexes was carried out using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, California) in a 10 cm plate as described by the manufacturer. The cells were transfected in media containing no antibiotics and having a final concentration of RNA duplex of 25 nM as described above. Cells were incubated for 48 h before harvesting and immunoprecipitation (IP) of c-myc Ago2. Cell harvest and IP was performed as previously described (9,13). Cells were collected from plates and washed once with 1× PBS and once in 2 ml of hypotonic lysis buffer (HLB) [10 mM Tris pH 7.5, 10 mM KCl, 2 mM MgCl₂, 5 mM DTT and EDTA-free complete protease inhibitor cocktail (Roche, Basel, Switzerland)]. Cells were then reconstituted in 0.5 ml of HLB and allowed to swell on ice for 15 min. A fraction of the cell lysate (50 µl) was taken and added to 200 µl Trizol (Invitrogen, Carlsbad, California) for subsequent gene silencing assays using the QuantiGene bDNA hybridization assay (Panomics, Fremont, California). The remainder of the cell lysate was added to a 1 ml Dounce homogenizer with a tight pestle and cells were homogenized for 30 strokes on ice. Cell lysates were clarified by centrifugation (14 000 r.p.m., 30 min at 4°C) and supernatant was transferred to a new tube. One milliliter LB650 buffer (0.5% NP40, 150 mM NaCl, 2 mM MgCl₂, 2 mM CaCl₂, 20 mM Tris pH 7.5, 5 mM DTT, 650 mM KCl and EDTA-free complete protease inhibitor cocktail) was added to supernatant or cytosolic fraction. Anti-c-myc antibody conjugated to agarose beads (Sigma, St. Louis, Missouri) was added to each tube and the tubes were incubated overnight at 4°C while rotating. After overnight incubation, IP reactions were centrifuged for 2 min at 3000 r.p.m. and then beads were washed three times in LB650 buffer. After washing the beads, 200 µl of Trizol reagent was added to disassociate the RNA from the antibody captured Ago2. RNA was precipitated as described by manufacturer instructions forgoing an ethanol wash. RNA was reconstituted in 20 µl of TE buffer (10 mM Tris–HCl, 1 mM EDTA).

Northern blot of RNA captured from Ago2 immunoprecipitation

RNA was analyzed on a 15% polyacrylamide, TBE–urea denaturing gel. ³²P-end size makers ranging from 21 to 25 nt (see Supplementary Table S3 for sequences) were included on the gel to determine the size of the captured RNA. The RNA was transferred to a nylon membrane and UV cross-linked. The membrane was pre-hybridized using UltraHyb-Oligo Buffer (Ambion, Austin, Texas) for 30 min at 42°C and then ³²P-labeled DNA / locked nucleic acid probes complementary to the guide strand were added to the hybridization buffer. After overnight incubation at 42°C, the membrane was washed twice for 30 min in wash buffer (1× SSC buffer, 0.1% SDS). The blot was visualized by exposing to BioMAX autoradiography film (Kodak, Eastham, New York). Film was developed using an automatic film processing unit.

mRNA cleavage position assay

293T cells expressing c-myc Ago2 were transfected with RNA duplexes and Ago2 was immunoprecipitated as described above. After the final wash, the agarose beads containing Ago2 complexes loaded with transfected RNAs were re-constituted in 10 μ l buffer (100 mM KCl, 2 mM MgCl₂ and 10 mM Tris pH 7.5). A synthetic RNA identical to a 50-nt region of the human SOD1 gene (see Supplementary 3 for sequence) containing the target site for the transfected 25-bp duplexes or 19-bp + 2-nt siRNAs was ³²P-5'-end labeled and gel purified. The synthetic substrate was designed specifically to have base 21 correspond to position 10 of the guide strand of the 19-bp + 2-nt siRNA or 25-bp duplex. For the cleavage reaction, the reconstituted IP reaction (10 μ l) was added to 4 μ l of labeled synthetic substrate, 1 μ l RNAsin (Promega, Madison, Wisconsin) and 5 μ l buffer (100 mM KCl, 2 mM MgCl₂ and 10 mM Tris pH 7.5) for a final volume of 20 μ l. The reaction was incubated for 2 h at 30°C. After incubation, the reactions were analyzed on a 15% polyacrylamide, TBE-urea denaturing gel. The gel was exposed to autoradiography film as above. Size of cleavage product was determined by comparison to ³²P-labeled size marker RNAs.

Dicer processing assay

RNA duplexes were incubated with recombinant human Dicer enzyme (Genlantis, San Diego, California) according to manufacturer recommendations and previously described conditions (4). Samples were incubated overnight (~16 h) at 37°C with and without Dicer enzyme. Reactions were stopped by the addition of TBE gel loading buffer and snap frozen in liquid nitrogen. A fraction (16 pmol) of the sample was analyzed on a native, TBE buffered, 20% polyacrylamide gel. An siRNA marker (New England Biolabs, Ipswich, Massachusetts) was included on the gel. The gel was stained using SYBR green II (Invitrogen, Carlsbad, California) for 20 min and then visualized using a UV transilluminator and CCD camera. UV images and relative quantification analysis were carried out using the UVP BioChem imaging station with LabWorks software (UVP, Upland, California).

Generation of 100-bp dsRNA

The template for *in vitro* transcription to generate the 100-bp RNA was derived by high fidelity PCR amplification (Roche, Indianapolis, Indiana) of a mouse SOD1 cDNA clone (Accession number: BC048874) (Origene, Rockville, Maryland). The forward (TAATACGACTCACTATAGGGCGTGTGATCTCACTCT) and reverse (TAATACGACTCACTATAGGGTACTTTCTTCATTTCCACCTTTGC) primers (Integrated DNA Technologies, Coralville, Iowa) incorporate a T7 promoter on both strands. The PCR fragment was purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, California) and the size was verified on an agarose gel.

The *in vitro* transcription and purification of the 100-bp dsRNA was performed using the MEGAscript RNAi Kit (Ambion/Applied Biosystems, Austin, Texas) according to manufacturer's instructions.

Dicer null cell transfections

Mouse derived embryonic stem cells that are homozygous null or heterozygous for the enzyme Dicer were generated by Murchison and co-workers and cultured as previously described (12). Dose response transfections were carried out by adapting previously described conditions (14) to a 96-well dose response method described above. Active RNA duplexes targeting the human SOD1 gene that had 100% homology to mouse SOD1 were transfected in concentrations ranging from 0.05 to 10 nM in a final RNA concentration of 25 nM using RISC-free filler RNA (Dharmacon, Lafayette, Colorado). Cells were incubated for 48 h after transfection and mRNA levels were measured using the QuantiGene bDNA hybridization assay (Panomics, Fremont, California) as described above.

RESULTS

Modified RNA duplexes demonstrate potent RNAi activity

To explore how chemical modifications would affect RNAi activity we created a panel of 25-bp duplexes with several modification patterns and measured their ability to silence the superoxide dismutase gene (SOD1) (data not shown). We identified modification schemes that maintained efficient gene silencing when compared to an unmodified duplex. One such pattern consists of four 2'OME modifications on both ends of the passenger strand with the guide strand left unmodified, referred to throughout as modification pattern '4/4' (Figure 1A). Target mRNA levels were determined following transfection of human HEK293 cells with 19-bp + 2-nt 3'-end overhang (19-bp + 2-nt siRNA) and blunt-ended 25-bp duplex RNA either unmodified or with 4/4 2'OME modification (Figure 1B). The EC₅₀ (effective concentration required to induce a 50% effect) of the 25-bp modified RNA duplex was in the picomolar concentration range and equivalent to the silencing activity of the unmodified 25-bp duplex or 19-bp + 2-nt siRNA of the same sequence. This result is not sequence specific, potent activity is also observed when comparing modified and unmodified duplexes targeting other sites in SOD1 (data not shown). Furthermore, picomolar inhibition was also achieved by targeting the cyclophilin B (PPIB) gene in HEK293 cells with a 25 bp modified RNA duplex at a known site for gene silencing activity (Figure 1C). Gene silencing with these duplexes is not specific to human cells as similar potency is observed in murine (NIH3T3) cells (Supplementary Figure S1). Overall, we have identified duplexes with potency in the picomolar range for over 10 genes and at multiple sites per gene (data not shown).

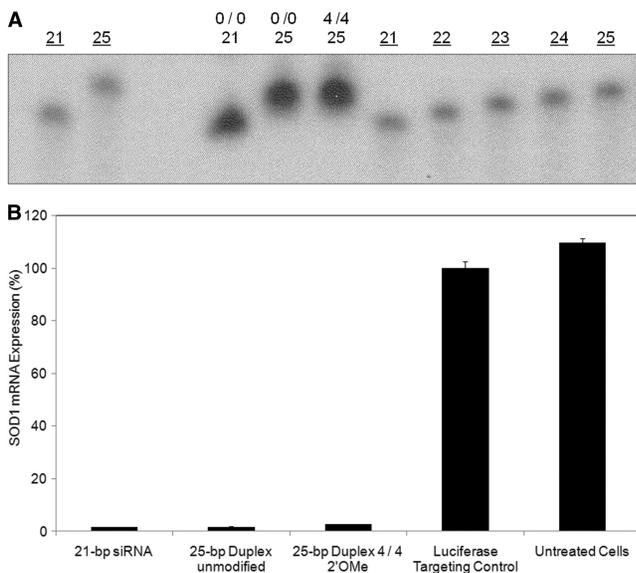


Figure 2. Northern blot detection of transfected RNA in Ago2. 293T cells expressing c-myc Ago2 were transfected with various duplexes that target the same area in SOD1. The cells were harvested, lysed, and c-myc Ago2 and associated RNA was immunoprecipitated as described in 'Materials and Methods' section. After immunoprecipitation, the RNA from the IP fractions was extracted and precipitated. RNA was loaded onto a denaturing polyacrylamide gel, transferred to a nylon membrane, and detected using a LNA probe specific to the guide strands of the transfected RNA duplexes. (A) Northern blot of the guide strand of SOD1 targeting RNA duplexes. ssRNA size markers 21–25-nt long (designated by underline) were included in lanes adjacent to the captured guide strand RNA (lanes: 21, 0/0; 25, 0/0; and 25, 4/4). (B) Expression of SOD1 after transfection of 293T cells expressing c-myc Ago2. Before immunoprecipitation of c-myc Ago2 a fraction of the cell lysate was taken for total cell RNA purification. The RNA was purified and gene expression was measured using a bDNA assay as described in 'Materials and Methods' section.

Supplementary Figure S3B). These results were extended to show that the full-length, unprocessed guide strand of a modified 26-bp duplex is also loaded into Ago2:RISC (Supplementary Figure S2).

Chemically modified RNA duplexes cleave the mRNA target at a defined position

Further characterization of the mechanism of action of a modified 25-bp duplex was carried out to determine the cleavage position(s) in the target sequence. It has been previously shown that classical siRNA will direct cleavage across from nucleotides 10 and 11 in reference to the 5'-end of the guide strand (9,15). There are two methods that are well suited to elucidate the position of mRNA target cleavage: 5' RACE PCR and an *in vitro* cleavage assay with immunopurified RISC on defined substrates. We chose to use immunopurified RISC to directly determine the nucleotide position of the cleavage site on a carefully designed synthetic substrate. To this end, a 50-nt, ³²Phosphate (³²P) radiolabeled substrate RNA was designed to mimic the target site of these active duplexes (Figure 3A). For the classical, 19-bp siRNA duplex, cleavage at the expected site in this synthetic substrate generates a 21-nt end-labeled product.

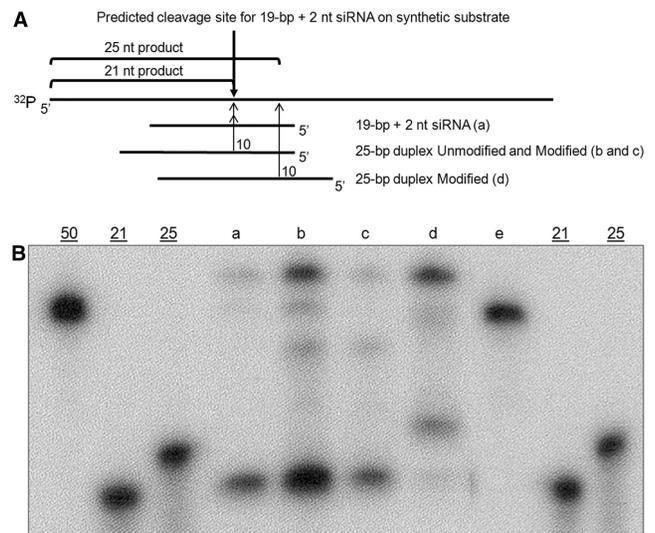


Figure 3. Modified RNA duplex (25 bp) cleaves target mRNA adjacent to positions 10–11 of the guide strand. (A) Schematic of the ³²P-labeled synthetic substrate, predicted cleavage positions and ³²P-labeled resultant products. (B) RNA duplexes targeting SOD1 were transfected into 293 cells expressing c-myc Ago2 and prepared as described in 'Materials and Methods' section for the *in vitro* cleavage reaction. Samples (a–e) were loaded onto a denaturing, polyacrylamide gel along with single-stranded RNA size markers of 21 and 25 nt and the full-length substrate (designated by underlines). Lanes a–e correspond to treatment with the following duplexes as shown in Figure 3A. a, unmodified 19-bp + 2-nt siRNA; b, 0/0 25-bp duplex; c, 4/4 2'OMe 25-bp duplex; d, 4/4 2'OMe 25-bp duplex, shifted 4 nt compared with b and c; e, Luciferase control duplex. Denaturation conditions were applied to the samples when running the PAGE, however, there seems to be a very strong interaction between actively targeting Ago2:RISC and the synthetic substrate (compared to lane e that does not target the synthetic substrate) which results in a band larger than the 50-nt substrate alone.

One chemically modified 25-bp duplex (c) was designed to have the same 5'-end as the siRNA; the other 25-bp duplex sequence is shifted by 4 nt (d). After transfection with the duplexes described above, Ago2 complexes were generated by immunoprecipitation (as described in Figure 2). The immunoprecipitates were incubated with the labeled synthetic substrate and the resultant cleavage products are shown in Figure 3B. The 25-bp duplexes (b and c) give rise to products that are the same size as the 19-bp + 2-nt siRNA (a), confirming that target cleavage occurs across from positions 10 and 11 relative to the 5'-end of the guide strand in Ago2. Re-positioning of 10 nt by using a targeting duplex with a different start position results in a concomitant shift in the cleavage site (as shown with duplex d) (Figure 3A). The result was confirmed with three other duplexes designed to direct cleavage in a different region of the SOD1 gene (Supplementary Figure S4). These results confirm that the position of the target cleavage site is defined by the 5'-end of the guide strand for RNA duplexes that are >19-bp in length.

Chemical modifications prevent Dicer processing

The above studies show that modified 25-bp RNA duplexes are able to achieve potent gene silencing, load

cell model system (Figure 5A). Our results were consistent with previous reports (12) and suggest that although longer RNAs can associate with Ago2 in the Dicer null cells, there appears to be a loss in efficacy when the RNA duplex is >39-bp. The EC₅₀ value doubles with a 41-bp duplex in the Dicer null cells when compared to the Dicer heterozygous cells. 100-bp duplexes silenced effectively in Dicer (+/–) cells but in the Dicer (–/–) cells there was extensive cell death at 24 h and there were no healthy adherent cells by 48 h after transfection making it impossible to accurately measure silencing activity

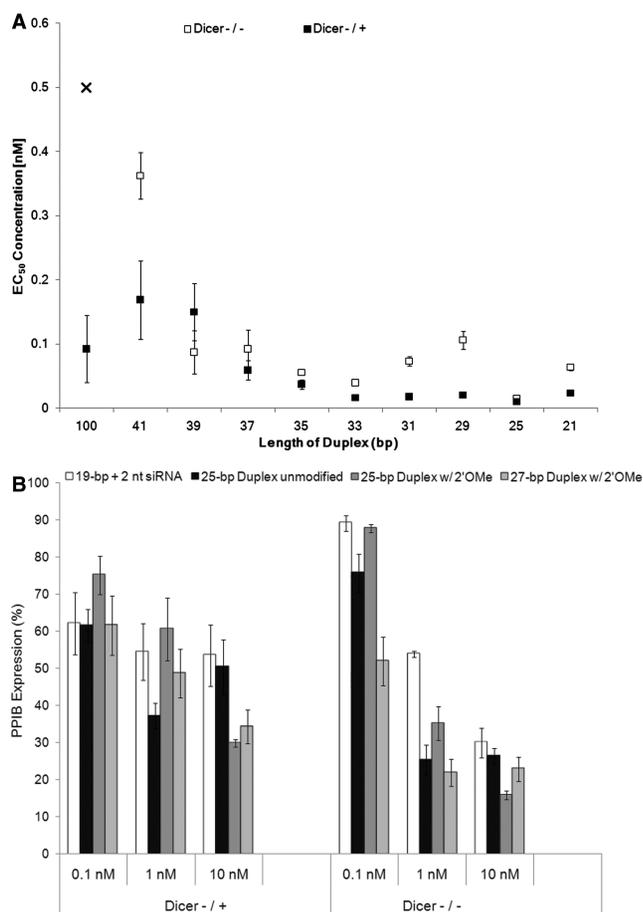


Figure 5. Dicer enzyme is not required for RNAi activity with RNA duplexes >21-bp. Mouse ES cells that were heterozygous (+/–) or homozygous null (–/–) for the Dicer gene were transfected with different RNA duplexes targeting SOD1 or PPIB as described in ‘Materials and Methods’ section. (A) EC₅₀ analysis of unmodified RNA duplexes targeting SOD1 ranging from 41 to 21 bp in length. The EC₅₀ values were calculated and generated from a dose response transfection as described in ‘Materials and Methods’ section. The EC₅₀ values were calculated using RNA duplex concentrations from 0.01 to 10 nM; Dicer heterozygous null cells (black squares), Dicer homozygous null cells (open squares). The EC₅₀ value for the 100-bp duplex in Dicer homozygous null cells (black X) was not determined due to significant cellular toxicity. (B) PPIB mRNA expression data for four duplexes are shown (in order); 19-bp + 2-nt siRNA (solid white bar), unmodified (0/0) 25-bp duplex (solid black bar), 4/4 2'OMe 25-bp duplex (dark gray bar) and 4/4 2'OMe 27-bp duplex (light gray bar).

(Supplementary Figure S7). These data support the biochemical results presented above showing that long, chemically modified duplexes are able to efficiently silence in the absence of Dicer (Figure 5B).

DISCUSSION

Chemical modification of RNAi duplexes has been demonstrated to impart novel properties that have benefits for efficacy *in vivo* and for therapeutic applications. The 2'OMe RNA nucleotide is one example of a chemical modification that has been shown to confer these attributes when applied to RNA duplexes in specific configurations. Incorporation of 2'OMe modifications can dramatically increase nuclease resistance in serum rich environments as compared to unmodified dsRNA (20). Depending on the configuration, as few as two 2'OMe modifications can prevent immune stimulation mediated through TLR-7 pathways (7). Jackson and colleagues showed that a single 2'OMe modification in the guide strand can reduce the number of off-target effects as demonstrated through microarray analyses (8). Addition of 2'OMe modifications to the passenger strand can also inhibit incorporation of this strand into the Ago2:RISC thereby reducing off-target effects. There are two potential ways that a 2'OMe modification could block incorporation into Ago2:RISC; inhibition of phosphorylation and prevention of interaction with the protein. Liu and colleagues have shown that phosphorylation of the 5'-end is required for an RNA strand to interact with Ago2 (9) since there is a binding site for the phosphate in the protein's MID domain (9,10). Furthermore, Nykänen and co-workers demonstrate that 2'OMe modification of the 5' most nucleotide blocks phosphorylation activity at this position (21). The 2'OMe modification is also observed to prevent interaction of the passenger strand with Ago2:RISC when compared to an unmodified passenger, even when this strand has been exogenously phosphorylated (Supplementary Figure S6). Since the discovery that long dsRNA is a mediator of RNAi, novel nucleic acid structures with a large variety of chemical modifications and nucleotide configurations have been tested for silencing potency as well as other therapeutic properties. Our studies were designed to address the mechanism of action of longer, 2'OMe modified RNA duplexes in comparison to the extensively investigated mechanism of action for classical siRNA (9).

Data presented here expand the understanding of how chemically modified RNA duplexes interact with key proteins involved in the silencing pathway. Although there have been previous mechanism of action studies looking at Dicer processing *in vitro* (4,5,22) these studies further characterize the impact of nucleotide modification on RNAi activity. These investigations probed multiple aspects of the RNAi mechanism of action such as RISC loading, cleavage of the target mRNA, activity in the absence of Dicer, *in vitro* Dicer enzyme processing and gene silencing activity. Models have been proposed suggesting that the enzyme Dicer is necessary for, or enhances, RISC-mediated gene silencing (4). It is

undisputed that Dicer is required for miRNA and shRNA processing and is required to trigger RNAi by very long, RNA duplexes, however, its role in RNAi with exogenous RNA is still not clear. Dicer acts as a molecular ruler by cleaving long stretches of RNA duplex into smaller species of ~21-bp in length (23). In the case of the chemical modification patterns tested, we show that 2'OMe modifications properly positioned can prevent Dicer processing of dsRNA that are long enough to serve as a Dicer substrate, yet these unprocessed RNAs still maintain silencing potency (Figure 1 and Supplementary Figure S1). In addition, potency of longer duplexes is maintained in cells that lack the Dicer enzyme (Figure 5).

Studies by MacRae and colleagues support the ability of exogenous duplexes to enter the RNAi pathway with no Dicer requirement as there was no co-association between Ago2 and Dicer observed when reconstituting RISC loading *in vitro* (11). Alternatively, some models do propose that Dicer transports or loads the RNA duplex into Ago2:RISC, where passenger strand separation is directed by a Dicer associated protein or Ago2 itself. To date, the mechanism of action of RISC loading remains a subject of examination. It was first proposed by Tomari and colleagues that a protein sensor (Dicer) carries out helicase activity to disassociate the passenger strand when associated with Ago2 (24). Further study led to a second model that strongly suggested that the RNA duplex is presented into RISC and the passenger strand is instead cleaved before removal by Ago2 (25). Both mechanisms (unwinding or cleavage) may be operative. The 2'OMe modification, when positioned in certain locations on the passenger strand, can drastically reduce or abolish RNAi activity possibly due to the inability of the passenger strand to be dissociated [data not shown and (15,26)]. Several proteins have been implicated in the RISC loading mechanism of action but are not always in agreement when comparing *in vitro* reconstitutions to cell-based manipulations of the RNAi mechanism or when comparing studies in different organisms (11,12,26–31). The data presented here demonstrate that Dicer cleavage activity is not essential for triggering of RNAi activity with exogenous RNA duplexes although the Dicer protein may still play a role in RISC loading. It is also possible that other dsRNA-binding proteins may play a key role in RISC loading such as the recently reported TAR RNA-binding protein (TRBP) in mammalian cells (27,30).

The observation that modified duplexes not cleaved by Dicer still function as very potent gene silencers, led us to investigate other aspects of their interaction with RISC. Immunoprecipitation of Ago2-associated RNAs allowed us to establish that the guide strand is loaded into the Ago2:RISC as a full-length, unprocessed product (Figure 2 and Supplementary Figures S2 and S3) and demonstrated that these duplexes are resistant to Dicer processing (Figure 4 and Supplementary Figure S5). Interestingly, we identified one particular 25-bp SOD1 targeting duplex that is efficiently processed by the Dicer enzyme *in vitro* (Figure 4), but is not processed in the cells (Figures 2 and 3). This result suggests some sequence specific affinity for RNAi-associated proteins

that circumvent Dicer and could have implications for the mechanism of gene silencing *in vivo*. Our findings demonstrate that longer duplexes load into the RISC complex in a manner which results in a target cleavage position defined by the 5'-end of the RNA guide strand. These results are consistent with a recent X-ray crystal structure of an Ago2-nucleotide complex showing that longer guide strands can be accommodated as well as reports examining the guide strand/mRNA interaction through crystal structure and cleavage position analyses (32,33).

RNAi has been shown to be a conserved process that will require further investigation to define details of the full mechanism of action. This study of the RNAi mechanism with modified duplexes demonstrates that there can be some generalization of the information obtained from studies with classical 19–21-bp siRNAs. In particular, we have shed light on the structural features that lead to positioning of the guide strand in RISC and definition of the seed region sequence for longer, chemically modified RNA duplexes. These results expand upon the current model of RNAi induced by synthetic dsRNAs by extending recent observations regarding Dicer and the active RISC. Chemical modification of dsRNA provides many advantages for the further development of RNAi compounds as therapeutics and further elucidation of the pathway with such reagents should enable improved RNA compound design.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

A special thanks to Greg Hannon for his helpful advice in designing experiments, reviewing data and assistance in the preparation of this manuscript. Craig Mello and Anastasia Khvorova are thanked for providing helpful discussion in reviewing the results and manuscript. We thank Fedor Karginov, Elizabeth Murchison, Sihem Cheloufi and Krista Marran for their time in providing technical advice. Additionally, we would like to acknowledge the staff of RXi Pharmaceuticals for their helpful discussions and support in the preparation of this manuscript.

FUNDING

Funding for open access charge: RXi Pharmaceuticals Corporation.

Conflict of interest statement. All authors are employees or consultants of RXi Pharmaceuticals and possess RXi stock.

REFERENCES

1. Chu, C.Y. and Rana, T.M. (2007) Small RNAs: regulators and guardians of the genome. *J. Cell. Physiol.*, **213**, 412–419.

2. Elbashir, S.M., Lendeckel, W. and Tuschl, T. (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.*, **15**, 188–200.
3. Carstea, E.D., Hough, S., Wiederholt, K. and Welch, P.J. (2005) State-of-the-art modified RNAi compounds for therapeutics. *IDrugs*, **8**, 642–647.
4. Kim, D.H., Behlke, M.A., Rose, S.D., Chang, M.S., Choi, S. and Rossi, J.J. (2005) Synthetic dsRNA dicer substrates enhance RNAi potency and efficacy. *Nat. Biotechnol.*, **23**, 222–226.
5. Kubo, T., Zhelev, Z., Ohba, H. and Bakalova, R. (2007) Modified 27-nt dsRNAs with dramatically enhanced stability in serum and long-term RNAi activity. *Oligonucleotides*, **17**, 445–464.
6. Chiu, Y.L. and Rana, T.M. (2003) siRNA function in RNAi: a chemical modification analysis. *RNA*, **9**, 1034–1048.
7. Robbins, M., Judge, A., Liang, L., McClintock, K., Yaworski, E. and MacLachlan, I. (2007) 2'-O-methyl-modified RNAs act as TLR7 antagonists. *Mol. Ther.*, **15**, 1663–1669.
8. Jackson, A.L., Burchard, J., Leake, D., Reynolds, A., Schelter, J., Guo, J., Johnson, J.M., Lim, L., Karpilow, J., Nichols, K. et al. (2006) Position-specific chemical modification of siRNAs reduces “off-target” transcript silencing. *RNA*, **12**, 1197–1205.
9. Liu, J., Carmell, M.A., Rivas, F.V., Marsden, C.G., Thomson, J.M., Song, J.J., Hammond, S.M., Joshua-Tor, L. and Hannon, G.J. (2004) Argonaute2 is the catalytic engine of mammalian RNAi. *Science*, **305**, 1437–1441.
10. Hutvagner, G. and Simard, M.J. (2008) Argonaute proteins: key players in RNA silencing. *Nat. Rev. Mol. Cell Biol.*, **9**, 22–32.
11. MacRae, I.J., Ma, E., Zhou, M., Robinson, C.V. and Doudna, J.A. (2008) In vitro reconstitution of the human RISC-loading complex. *Proc. Natl Acad. Sci. USA*, **105**, 512–517.
12. Murchison, E.P., Partridge, J.F., Tam, O.H., Cheloufi, S. and Hannon, G.J. (2005) Characterization of dicer-deficient murine embryonic stem cells. *Proc. Natl Acad. Sci. USA*, **102**, 12135–12140.
13. Caudy, A.A., Myers, M., Hannon, G.J. and Hammond, S.M. (2002) Fragile X-related protein and VIG associate with the RNA interference machinery. *Genes Dev.*, **16**, 2491–2496.
14. Schaniel, C., Li, F., Schafer, X.L., Moore, T., Lemischka, I.R. and Paddison, P.J. (2006) Delivery of short hairpin RNAs—triggers of gene silencing—into mouse embryonic stem cells. *Nat. Methods*, **3**, 397–400.
15. Elbashir, S.M., Martinez, J., Patkaniowska, A., Lendeckel, W. and Tuschl, T. (2001) Functional anatomy of siRNAs for mediating efficient RNAi in drosophila melanogaster embryo lysate. *EMBO J.*, **20**, 6877–6888.
16. Kessler, C. and Manta, V. (1990) Specificity of restriction endonucleases and DNA modification methyltransferases a review (edition 3). *Gene*, **92**, 1–248.
17. Yoshinari, K., Miyagishi, M. and Taira, K. (2004) Effects on RNAi of the tight structure, sequence and position of the targeted region. *Nucleic Acids Res.*, **32**, 691–699.
18. Kanellopoulou, C., Muljo, S.A., Kung, A.L., Ganesan, S., Drapkin, R., Jenuwein, T., Livingston, D.M. and Rajewsky, K. (2005) Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. *Genes Dev.*, **19**, 489–501.
19. Bernstein, E., Kim, S.Y., Carmell, M.A., Murchison, E.P., Alcorn, H., Li, M.Z., Mills, A.A., Elledge, S.J., Anderson, K.V. and Hannon, G.J. (2003) Dicer is essential for mouse development. *Nat. Genet.*, **35**, 215–217.
20. Choung, S., Kim, Y.J., Kim, S., Park, H.O. and Choi, Y.C. (2006) Chemical modification of siRNAs to improve serum stability without loss of efficacy. *Biochem. Biophys. Res. Commun.*, **342**, 919–927.
21. Nykanen, A., Haley, B. and Zamore, P.D. (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell*, **107**, 309–321.
22. Kubo, T., Zhelev, Z., Ohba, H. and Bakalova, R. (2008) Chemically modified symmetric and asymmetric duplex RNAs: an enhanced stability to nuclease degradation and gene silencing effect. *Biochem. Biophys. Res. Commun.*, **365**, 54–61.
23. Du, Z., Lee, J.K., Tjhen, R., Stroud, R.M. and James, T.L. (2008) Structural and biochemical insights into the dicing mechanism of mouse dicer: a conserved lysine is critical for dsRNA cleavage. *Proc. Natl Acad. Sci. USA*, **105**, 2391–2396.
24. Tomari, Y., Matranga, C., Haley, B., Martinez, N. and Zamore, P.D. (2004) A protein sensor for siRNA asymmetry. *Science*, **306**, 1377–1380.
25. Matranga, C., Tomari, Y., Shin, C., Bartel, D.P. and Zamore, P.D. (2005) Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell*, **123**, 607–620.
26. Meister, G., Landthaler, M., Dorsett, Y. and Tuschl, T. (2004) Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing. *RNA*, **10**, 544–550.
27. Doi, N., Zenno, S., Ueda, R., Ohki-Hamazaki, H., Ui-Tei, K. and Saigo, K. (2003) Short-interfering-RNA-mediated gene silencing in mammalian cells requires dicer and eIF2C translation initiation factors. *Curr. Biol.*, **13**, 41–46.
28. Lee, Y., Hur, I., Park, S.Y., Kim, Y.K., Suh, M.R. and Kim, V.N. (2006) The role of PACT in the RNA silencing pathway. *EMBO J.*, **25**, 522–532.
29. Robb, G.B. and Rana, T.M. (2007) RNA helicase A interacts with RISC in human cells and functions in RISC loading. *Mol. Cell*, **26**, 523–537.
30. Daniels, S.M., Melendez-Pena, C.E., Scarborough, R.J., Daher, A., Christensen, H.S., El Far, M., Purcell, D.F., Laine, S. and Gagnon, A. (2009) Characterization of the TRBP domain required for dicer interaction and function in RNA interference. *BMC Mol. Biol.*, **10**, 38.
31. Lima, W.F., Wu, H., Nichols, J.G., Sun, H., Murray, H.M. and Crooke, S.T. (2009) The binding and cleavage specificities of human Argonaute2. *J. Biol. Chem.*, **284**, 26017–26028.
32. Wang, Y., Sheng, G., Juranek, S., Tuschl, T. and Patel, D.J. (2008) Structure of the guide-strand-containing argonaute silencing complex. *Nature*, **456**, 209–213.
33. Wang, Y., Juranek, S., Li, H., Sheng, G., Tuschl, T. and Patel, D.J. (2008) Structure of an argonaute silencing complex with a seed-containing guide DNA and target RNA duplex. *Nature*, **456**, 921–926.