First step in pre-miRNAs processing by human Dicer

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Aim: To investigate the strand preference of the initial cleavage of human pre-miRNAs by human Dicer in vitro.

Methods: We used a series of in vitro transcribed pre-miRNAs that were radioactively labeled at their 5’ or 3’ ends in cleavage reactions with recombinant human Dicer or HeLa cytoplasmic S100 extracts. Pre-miRNAs samples were purified by denaturing and native PAGE and only the stem-loop structures were used in the experiments. Products of cleavage reactions were resolved by denaturing PAGE, and scanned by phosphor-imaging.

Results: Recombinant hDicer performs a biased first-cleavage in the pre-let-7b and hsa-pre-miR-17 3’ strand. This result is recapitulated in HeLa S100 cytoplasmic extracts.

Conclusion: The differential first-nick is observed in cleavage reactions only when stem-loops are substrates for hDicer.

Keywords: human Dicer; hsa-pre-miRNA; stem-loop; in vitro; first-nick

Original Article

Introduction

In mammals, microRNA (miRNAs) biogenesis begins when primary microRNAs (pri-miRNAs) are processed by the microprocessor complex (Drosha/DGCR8) to form ~60–80 nt hairpin-like precursors (pre-miRNAs) inside the nucleus. These precursors are subsequently exported to the cytoplasm via exportin 5 [1–3], where they are further processed by Dicer, a type III RNase that cleaves the stem-loop to yield shorter, double-stranded miRNAs (duplex miRNAs) with characteristic 2 nt 3’ overhangs, 3’ OH groups and 5’ phosphates [4, 5].

Human Dicer (hDicer) is a ~220 kDa protein that contains an N-terminal DExH-box RNA helicase-like domain, a domain of unknown function (DUF283), a PIWI-Argonaute-Zwille (PAZ) domain, two RNAse-III domains (RIIa and RIIib), and a double stranded RNA binding domain (dsRBD) [6]. Biochemical and crystallographic analyses of hDicer indicate that Mg²⁺, but not ATP, is required for RNA cleavage [7–9]. Analysis of dsRNA processing by hDicer enzymes that are engineered with specific RIII domain mutations revealed that hDicer contains a single dsRNA processing center, which is formed via intramolecular dimerization of the two RIII domains; these domains function independently to cleave phosphodiester bonds on opposite strands of the dsRNA substrate [8, 10]. hDicer has been shown to associate with the human immunodeficiency virus transactivating response RNA-binding protein (TRBP) to process pre-miRNAs.

Despite our current knowledge of hDicer biochemistry and its activity on perfectly matched double-stranded RNAs, little is known about the first step in the pre-miRNA processing. Some studies have used radioactive pre-miRNAs to show that when hDicer finds and cleaves a pre-miRNA, cleavage does not occur simultaneously in both strands [11, 12] due to the fact that Dicer contains one catalytic center. In those studies, an intermediate band (~50 nt) between the intact pre-miRNA and the mature miRNA was visualized, suggesting that hDicer cleaves (or makes a first-nick in) a specific strand. However, it is not clear whether hDicer cleaves the 5’ or 3’ strand preferentially, or whether its preference is dependent on the substrate. Using a series of precursor stem-loops and differential labeling, we show that recombinant hDicer makes a first-nick in a specific strand and that this activity is recapitulated in S100 HeLa cytoplasmic extracts. We also show that 3’ overhangs influence the strand selection for the first-nick. Our in vitro results suggest that hDicer selects the pre-miRNA strand by first positioning the 3’ overhang.
Materials and methods
DNA templates for in vitro transcription
DNA oligonucleotides were chemically synthesized by SIGMA/Genosys (St Louis, MO) or IDT (Coralville, IA) and purified by PAGE. Each oligonucleotide contains a DNA sequence complementary to the microRNA precursor sequences and to the sequence of T7 RNA polymerase promoter at the 3’ end (Table 1). The double-stranded templates for in vitro transcription were prepared with the Klenow fragment of DNA polymerase I. The DNA template reaction for each precursor contained 6 µL of 50 µmol/L T7 primer, 6 µL of 50 µmol/L precursor oligonucleotide, 20 µL 10×Klenow reaction buffer, 5 µL of 10 µmol/L dNTPs and 158 µL of water. The mixture was heated for 2 min at 94 ºC and slowly cooled to 37 ºC. Once cooled, 2 units of Klenow enzyme were added and incubated for 1 h at 37 ºC. Finally, the reactions were phenol/chloroform extracted and diluted in 50 µL of DEPC-treated water.

In vitro transcription and RNA purification
The pre-microRNAs used in this study were prepared by in vitro transcription with T7 RNA polymerase (New England Biolabs, Ipswich, MA). Transcription reactions were carried out in a 120 µL volume containing 150 pmol of DNA template, 4 µL of each rNTP at 100 mmol/L, 20 µL of MgCl₂ at 50 mmol/L, 12 µL of 10×T7 reaction buffer and 500 units of T7 RNA polymerase. The transcription reactions were incubated at 37 ºC overnight. [α-³²P] UTP was added by the T7 transcription. Transcripts were run on denaturing 8 mol/L urea, 10% PAGE (acrylamide/bisacrylamide, 19/1) gels in 0.5×TBE, gel-excised, eluted from the gel in 0.3 mol/L sodium acetate, pH 5.2, 0.5 mmol/L EDTA, and 0.1% SDS, and precipitated with ethanol. All transcripts were phosphorylated at the 5’-end by T4 polynucleotide kinase with cold or [γ-³²P] ATP (4500 Ci/mmol; Amersham) and phenol/chloroform extracted. For 3’ end labeled RNAs, RNA ligase (Ambion, Austin, TX) and [α-³²P] pCp were used according to the manufacturer’s recommendations. As pCp labeling leaves a 3’ phosphate, we removed the extra phosphate using either T4 kinase (New England Biolabs, Ipswich, MA) or alkaline phosphatase. The samples were then phenol/chloroform extracted, precipitated, and subjected to another round of 5’ labeling with cold ATP.

Stem-loop structure purification by native PAGE
We analyzed the RNA structure homogeneity of all investigated transcripts by native 10% PAGE (acylamide/bisacrylamide, 19/1) in 0.5×TBE. Only the bands containing hairpin-like structures were excised, eluted overnight and ethanol precipitated. To prevent the disruption of the stem-loop structures, the samples were maintained at 4 ºC until the experiments with the Dicer enzyme were carried out. Secondary structures were predicted using the Mfold algorithm (release 3.2)[14].

Hsa-pre-miR-17 5’ and 3’ end mapping
The ends of hsa-pre-miR-17 were identified according to the procedure used previously for human let-7 precursors[15]. Briefly, total RNA was obtained from HeLa cells using Trizol (Ambion, Austin, TX), and the ≤100 nt fraction was collected using FlashPage electrophoresis (Ambion, Austin, TX). The RNA was self-ligated with T4 RNA ligase. Following ligation, the RNA was phenol/chloroform extracted and precipitated with ethanol. The hsa-pre-miR-17 sequence was amplified by RT-PCR using specific hsa-pre-miR-17 primers (Table 1). PCR products were resolved in a 4% LMP agarose gel (Invitrogen, Carlsbad, CA) and the band was excised, column-purified (Invitrogen, Carlsbad CA) and cloned with the pDrive cloning kit (Qiagen). Plasmid DNA from seven individual clones derived from three independent RNA extractions were sequenced and found to be identical (data not shown).

Table 1. DNA oligonucleotides.

| Oligonucleotide          | Sequence (5’–3’)          |
|-------------------------|---------------------------|
| Hsa-Pre-let-7a-2        | GGAAGCGTACGGGCTGTACATTCTCCCTTGATGTAATTCTAAACTATACAACTACCTACACTACCTACGATGCTATTA |
| Hsa-Pre-let-7b          | GGGAGGCGATGTGGTTATGGTTATTTCTCCGAGGAGGAGACATCTGCCCTGAAACACAACAAACACTACCTACATAGTGAGTCGTATTA |
| Hsa-Pre-miR-30a         | GCTCGAAACATCGACTGAAGGCTCCTACGCTCACTTCTCCAGGAGTGGTTTCACACTAGTAGTGAGTCGTATTA |
| Hsa-Pre-miR-17          | GCTACAAGTGGCTCCTACGAGTAGAGTGCACATATACACTACCTACGCTGAGTGAAGCTATGAGTCGTATTA |
| Hsa-pre-miR-17, 5’ overhang | AAGCTTAGGAGGCTGTACATTCTCCCTTGATGTAATTCTAAACTATACAAACACTACCTACATAGTGAGTCGTATTA |
| Hsa-pre-miR-17, 5’ overhang | AAGCTAGGAGGCTGTACATTCTCCCTTGATGTAATTCTAAACTATACAAACACTACCTACATAGTGAGTCGTATTA |
| Hsa-pre-miR-17, 5’ overhang | TGAACAATCGCTGAGTAAAGCCCATCTTGGGTTCCATAGCTCGTTGAGGAGTGGTTTTACACTAGTAGTGAGTCGTATTA |
| Hsa-pre-miR-17, 5’ overhang | TTAGCTACAGCTATAG |
| Hsa-pre-miR-17 forward  | CTATTTGACGATATG |
| (to map the 5’ and 3’ ends) | (to map the 5’ and 3’ ends) |
In vitro reactions using hDicer and cytoplasmic HeLa extracts

For in vitro reactions using recombinant hDicer, we incubated 5×10^4–10×10^4 cpm. of labeled precursor with 1 U of rDicer (Genlantis) according to the manufacturer’s instructions. For in vitro reactions using HeLa cell S100 cytoplasmic extracts (kindly provided by Phillip D ZAMORE, University of Massachusetts), we incubated 5×10^4–1×10^5 cpm. of labeled precursor with 25 μg of cell extract, 3.2 mmol/L MgCl_2 and 0.5 mmol/L ATP at 37 °C for the indicated times. Reactions were stopped by the addition of loading buffer containing 7.5 mol/L urea, heated at 95 °C for 5 min, and immediately run in 15%–20% denaturing PAGE. After electrophoresis, the gel containing the separated RNAs was exposed to a phosphor-screen and analyzed by phosphorimaging. The processing efficiency for each hairpin RNA (Figure 1) was determined as previously reported: \[ \text{processing efficiency} = \frac{\text{mature IDV}}{\text{mature IDV} + \text{precursor IDV}}. \]

IDV: Integrated Density Value.

Results

Precursor miRNAs used in this study

miRBase is the public database where the sequences of all newly discovered mature microRNAs are deposited for classification. However, precursor miRNAs (either pre-miRNAs or pri-miRNAs) are not annotated for actual sequences; the predicted stem-loop sequences in the database are not strictly pre-miRNAs, but include the pre-miRNA and some flanking sequence from the presumed primary transcript. Currently, ends have been mapped for only a few pre-miRNAs. For this reason, we mapped the 5’ and 3’ ends of hsa-pre-miR-17 from HeLa cells using a method reported previously (see Methods and Figure 1A). This pre-miRNA was initially reported to contain only one mature miRNA (miR-17, 23 nt), but another miRNA was later identified in the complementary strand (miR-17*, 22 nt). Notably, we found that pre-miR-17 contains an extra cytosine that is not found in the mature mir-17* sequence. Recent studies have reported variability in pre-miRNA ends due to Drosha processing, which may explain why we observed this extra nucleotide. We used this sequence as the actual pre-miR-17 throughout this study. In addition to pre-miR-17, we included three other pre-miRNAs, the ends of which have been mapped previously: hsa-pre-miR-30 (pre-miR-30a), hsa-pre-let-7a-2 (pre-let-7b), and hsa-pre-let-7b (pre-let-7b).

We aimed to determine whether differential cleavage occurs in the first step of cleavage by hDicer on the miRNA or miRNA* strands when hDicer finds a stem-loop. For this reason we double-purified all RNAs by denaturation and native PAGE (Figure 2). Following transcription, we purified the RNAs by denaturing PAGE and tested the samples for degradation and purity (Figure 2A). After this step, RNA molecules adopted one of two major structures, an RNA stem-loop or an RNA dimer (Figure 2B and 2C), which we then resolved by native PAGE (Figure 2D, left). Denaturing gel extraction did not entirely prevent RNA dimer formation, even when we heated the samples at 95 °C and slowly cooled them to RT. On the contrary, heating the samples promoted dimer formation. Therefore, we subsequently gel extracted only the stem-loops and verified their integrity by a subsequent native PAGE prior to each experiment (Figure 2D, right).

First-nick step in pre-miRNA processing by recombinant hDicer

We incubated internally labeled pre-miRNAs with recombinant hDicer and allowed the cleavage reactions to proceed until the indicated time points (Figure 3A–3D). All precursor RNAs were processed by hDicer to produce two bands near 20 nt (gray arrows), which correspond to mature miRNAs and might also correspond to a piece of or the entire loop sequence. The abundance of mature miRNAs increased in a time-dependent manner as a result of hDicer activity. In addition to the mature miRNAs, bands near ~40–50 nt were observed (Figure 3A–3D, black arrows). The length of the fragments obtained indicates that these bands correspond to a first-nick step in the processing that occurs near the loop of the pre-miRNAs. Interestingly, the first-nick intermediates decreased in abundance over the time course, in contrast to the mature miRNAs, which continued to accumulate for the entire incubation time (Figure 3A–3D, right graphs).

To test whether the first nick was made in the miRNA or miRNA* strand (namely 5’ or 3’ strand), we labeled either the 5’ or the 3’ end of each pre-miRNA and incubated the samples with hDicer (Figure 4A–4D). Time course experiments indicated that recombinant hDicer is able to discriminate the 5’ strand from the 3’ strand. First-nick intermediates for pre-let-7a-2, pre-let-7b and pre-miR-17 appeared to be labeled at the 5’ end (Figure 4A, 4B and 4D, asterisks), indicating that hDicer made the first nick in the 5’ strand of the stem-loop. In the case of pre-miR-30a, hDicer cleaved both strands with similar efficiency (Figure 4C, asterisks), indicating that recombinant hDicer can make the first nick on either strand. Next, we determined whether these results could be recapitulated in HeLa S100 cytoplasmic extracts. Pre-let-7b and pre-miR-17 were preferentially cleaved first in their 3’ strands (Figure 5B and 5D, asterisks); the 5’ strand of pre-let-7b was barely cleaved, and the first-nick in the 5’ strand of pre-miR-17 was negligible. Experiments with pre-let-7a-2 revealed a tendency to make the first nick on the 5’ strand. By contrast, pre-miR-30a was preferentially nicked on the 3’ strand (Figure 5A and 5C, asterisks). To determine the identity of the pre-let-7b and pre-miR-17 recombinant hDicer reaction products, we cloned and sequenced all reaction products from 19–60 nt (data not shown). This analysis revealed that first-nick intermediates possessed the 5’ strand and the loop in a 5:1 ratio. These results indicate that in vitro hDicer discriminates between strands when making the first nick, and that its choice of strand depends on the specific pre-miRNA. The first-nick bias does not seem to be related to the position of the mature miRNA or miRNA* (Figure 1A).

Since most pre-miRNAs are Drosha products generated in the nucleus, pre-miRNAs bear the characteristic signature of RNase III products: 2 nt, 3’ end overhangs. As it is known that the PAZ domain binds double-stranded RNA by anchoring 3’ end overhangs, we investigated whether...
the overhang position influenced the strand preference for the first-nick introduced by hDicer. We modified the ends of all pre-miRNAs tested to leave 2 nt 5’ overhangs (Figure 1B), incubated these modified pre-miRNAs with recombinant hDicer and resolved the reactions by denaturing PAGE (Figure 6). Our results indicate that 2 nt 5’ overhangs modified the first-nick strand preference, allowing the cleavage of both strands for pre-let-7b and pre-miR-17 (Figure 6B and 6D, asterisks) and biasing initial cleavage to the 5’ strand of pre-let-7a-2 and pre-miR-30a. Interestingly, pre-let-7b and pre-miR-30a intermediates changed in size when labeled at their 5’ or 3’ ends rather than possessing 3’ overhangs (Figures 4 and 5), indicating that the scissile phosphate was changed. These results suggest that hDicer anchors the PAZ domain as a first step in pre-miRNA cleavage, and therefore acts as a molecular ruler to cleave the precursor to a specific size by introducing the first-nick.

Figure 1. MicroRNA precursors used in this study. Secondary structures were predicted using Mfold (version 3.2)[1]. (A) Human miRNA precursors — pre-let-7a-2, pre-let-7b, and pre-miR-30a — 5’ and 3’ ends were used based on previous studies whereas human pre-mir-17 5’ and 3’ ends were identified in this work. (B) Precursors used in (A) having a 5’ overhang. To produce 5’ overhangs, indicated with grey boxes, we used the naturally nucleotides appearing in each primary precursor as templates (miRBase 12.0)[2–4] to overhang the 5’ ends, and cropped the 3’ ends leaving 2-nucleotide, 5’ overhangs. Nucleotides in red indicate mature miRNA sequences.
Discussion

Here, we show that hDicer differentiates between miRNA precursor strands and introduces a first-nick either in the 5’ or 3’ strand. The choice of first-nick strand can be modified by the presence of a 2 nt 5’ overhang in the precursor, supporting the model in which hDicer binds 3’ ends through its PAZ domain[6]. Our results suggest that, in order to act as a molecular ruler, hDicer initiates pre-miRNA processing after anchoring the 3’ end overhang. If improper binding of the 3’ overhang to the PAZ domain occurs, the processing of the pre-miRNA will be altered by a shift at the scissile phosphate (Figure 6B and 6D). This might be the result of the “wrong” positioning of the stem-loop in hDicer owing to the 5’ overhang. The stem loop adopts a helical shape[10], so the incorrect anchoring of the 5’ end could cause a shift in the cleavage site; a shift of two helical turns of the RNA would cause the observed ~22 nt product change. This in turn causes the intermediate to become longer or shorter. Lima et al reported recently that 3’ overhangs are important for the affinity of hDicer and siRNAs, as well as for biased strand loading into the RNA-induced silencing complex[29]. The erratic cleavage position of precursors with 5’ overhangs is interesting as none of these species has been reported endogenously. In light of our results, this suggests that the location of the first-nick might influence miRNA loading on Argonaute 2. Further studies will be required to verify this hypothesis.

Intermediates in pre-miRNAs processing have been visualized in denaturing gels previously[11, 13, 16, 29]; however, differential first-nicks have not been reported. We attribute our ability to see the differential first-nick to the gel-extraction and subsequent analysis of only the stem-loop precursor. Failure to perform this isolation step results in a mix of two major RNA structures in the samples — a stem loop and a dimer. Although the proportion of the RNA dimer is small compared with the stem loop (Figure 2C), the dimer is a substrate of hDicer. Consequently, no difference in the first nick could be visualized with a denaturing PAGE gel, as hDicer is able to enter from either end and cleave the RNA dimer (Figure 7A)
Therefore, a mix of dimer and stem-loop RNAs in the cleavage reaction masks the existence of the differential first-nick. This finding is particularly important because the standard method to form stem-loops is by heating the pre-miRNA samples; however, boiling the samples promotes RNA dimer formation.

Another interesting finding was that recombinant hDicer and HeLa cell extracts did not process pre-miRNAs identically. While a recombinant hDicer generated a differential first nick in pre-let-7b and pre-miR-17, we observed only a small bias towards one strand in pre-let-7a-2 and pre-miR-30a in HeLa cell extracts. This suggests that other pathway components, as well as their compartmentalization, might influence strand selection in endogenous pre-miRNA stem loops.

It will be important to investigate whether such differential first nick occurs in all human pre-miRNAs in living cells, as well as to identify other factors that might be involved in the processing.

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Author contribution
Carlos Fabián FLORES-JASSO designed and performed research, and wrote the paper. Catalina ARENAS-HUERTERO and Jose Luis REYES performed research and contributed ideas. Cecilia CONTRERAS-CUBAS performed research. Alejandra COVARRUBIAS contributed ideas and reagents.
Figure 4. Recombinant hDicer makes differential cleavages on pre-miRNAs. Pre-miRNAs were labeled radioactively either on their 5’ or 3’ ends and incubated with hDicer. Reactions were stopped at 5, 15, 30, 60, and 480 min and resolved by 20% (v/v) denaturing PAGE. Asterisks indicate the position of the first-nick product (~40–50 nt). hDicer cleaves pre-let-7a-2, pre-let-7b and pre-mir-17 stem-loops preferentially on one strand (3’ end strand), whereas pre-miR-30a is cleaved similarly on both strands. Arrows on the stem-loop drawings represent the preferential first-nick for each precursor. C, 0-time point; M, decade marker.

Figure 5. hDicer makes differential cleavages on pre-miRNAs in HeLa cytoplasmic extracts. Pre-miRNAs were labeled radioactively either on their 5’ or 3’ ends and incubated with HeLa cytoplasmic extracts. Reactions were stopped at 5, 15, 30, 60, and 480 min and resolved by 15% (v/v) denaturing PAGE. Asterisks indicate the position of the first-nick product (~40–50 nt). hDicer cleaves pre-let-7b and pre-mir-17 stem-loops preferentially on one strand (3’ end strand), whereas the first nick on pre-let-7a-2 — in contrast to our experiments using recombinant hDicer — is preferentially on the 5’ strand. There was a tendency towards the 3’ strand in pre-miR-30a. Arrow-sizes on the stem-loop drawings indicate the preferential first-nick for each precursor. C, 0-time point; M, decade marker.
Luis VACA contributed ideas and wrote the paper.

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