P68 RNA Helicase Unwinds the Human let-7 MicroRNA Precursor Duplex and Is Required for let-7-directed Silencing of Gene Expression*

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David W. Salzman, Jonathan Shubert-Coleman, and Henry Furneaux

From the Department of Molecular, Microbial, and Structural Biology, University of Connecticut Health Center, Farmington, Connecticut 06030

MicroRNAs are short, single-stranded RNAs that arise from a transient precursor duplex. We have identified a novel activity in HeLa cell extracts that can unwind the let-7 microRNA duplex. Using partially purified material, we have shown that microRNA helicase activity requires ATP and has a native molecular mass of ~68 kDa. Affinity purification of the unwinding activity revealed co-purification of P68 RNA helicase. Importantly, recombinant P68 RNA helicase was sufficient to unwind the let-7 duplex. Moreover, like its native homolog, P68 RNA helicase did not unwind an analogous small interfering RNA duplex. We further showed that knockdown of P68 inhibited let-7 microRNA function. From our data, we conclude that P68 RNA helicase is an essential component of the let-7 microRNA pathway, and in conjunction with other factors, it may play a role in the loading of let-7 microRNA into the silencing complex.

It is now recognized that microRNAs play a critical role in the regulation of gene expression and that their deregulation may underlie many human diseases (1–5). Thus, it is important to understand how they are incorporated into the protein cofactor complexes that are essential for their suppressive activity. MicroRNAs are initially transcribed as precursor RNAs, which fold into hairpin structures (6). The acknowledged first step of microRNA maturation is the specific endonucleolytic cleavage of the pri-microRNA near the base of the stem loop, by Drosha (7). The Drosha cleavage product is typically a 70-nucleotide stem loop RNA, containing a two-nucleotide overhang at the 3' end and a recessed 5'-phosphate (8–10). This product is a substrate for a second enzyme called Dicer (11). Dicer cleaves both strands of the hairpin precursor ~21 nucleotides from the end of the stem loop creating a 19-nucleotide paired duplex with two-nucleotide overhangs at the 3' ends: the transient microRNA duplex (12–14).

It is now clear that the silencing activities of microRNAs are effected by a novel class of RNA-binding proteins called the Argonaute family (15–18). However, the affinity of these proteins for duplex RNA is much less than that observed for single-stranded RNA (15, 19). Moreover, analysis of Argonaute/RNA complexes in the cell typically reveals the association of only one strand (the guide strand) of this transient duplex (20). Thus, there are likely to be additional factors, which unwind the transient duplex and confer specificity in uptake into Argonaute.

Studies on structurally different, but conceptually analogous, siRNA2 duplexes have revealed that this specificity in uptake may be conferred by a heterodimer of Dicer and R2D2 in which the stable end of the duplex is bound by R2D2 and Dicer facilitates the loading of the loosely paired 5’ end of the guide strand into Argonaute (21–24). Although the unwinding is likely facilitated by the cleavage of the passenger strand by Argonaute (25, 26), RNA helicase A has also recently been implicated in loading of the guide strand of siRNA (27). Much less is known about the factors that are likely required to load the guide strand of the analogous microRNA duplex into Argonaute complexes. In contrast to siRNA duplexes, microRNA duplexes typically contain unpaired bulges, which likely preclude the cleavage of the passenger strand by Argonaute (26).

An affinity-purified complex that contains Argonaute2 and Dicer has been shown to direct the cleavage of a target mRNA directed by a let-7 hairpin precursor (19, 28). Although this argues that Dicer might be necessary for the unwinding of the microRNA transient duplex, microRNA duplexes can still silence expression in cells that lack Dicer (29). In any event, neither the siRNA-directed nor the microRNA duplex-directed cleavage of a mRNA has been successfully reconstituted with recombinant proteins, and thus, it is likely that additional factors are required for this step.

In the present study, we have identified an activity from human cells that promotes the ATP-dependent unwinding of the human let-7 microRNA precursor duplex. Further characterization suggested that this activity corresponded to the previously described P68 RNA helicase (30–32). Indeed, we found that recombinant P68 RNA helicase was sufficient to unwind the let-7 microRNA precursor duplex. Importantly, a transient knockdown of P68 abrogated let-7-directed suppression of gene expression and indicates that P68 RNA helicase is indeed required to facilitate the uptake of duplex let-7 microRNA into the silencing complex.

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1 To whom correspondence should be addressed: University of Connecticut Health Center, 236 Farmington Ave., Farmington, CT 06030. Tel.: 860-679-2374; Fax: 860-679-1862; E-mail: furneaux@nso.uchc.edu.

2 The abbreviations used are: siRNA, small interfering RNA; GAPD, glyceraldehyde-3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AMP-PNP, adenosine 5’-(β,γ-imino)triphosphate.
EXPERIMENTAL PROCEDURES

HeLa S3 cells were obtained from the National Cell Culture Center (Minneapolis, MN). Synthetic RNAs and siRNAs were obtained from Dharmacon Research Inc. (Lafayette, CO). Luciferase reporter plasmids were provided by the David Bartel laboratory. The His-P68 plasmid was provided by the Zhi-Ren Liu laboratory. Anti-P68 monoclonal antibody (PAB204) was obtained from Upstate Biochemicals, and monoclonal antibodies against GAPD and Vimentin were obtained from Abcam.

Preparation of HeLa Cell Extract—HeLa S3 cells (National Cell Culture Center) were resuspended in hypotonic buffer (50 mM Tris, pH 7.5, 10 mM KCl, 5 mM dithiothreitol). The swollen cells were homogenized, and KCl, MgCl₂, and glycerol were added to final concentrations of 100 mM, 2 mM, and 10%, respectively. The homogenate was centrifuged at 10,000 × g for 10 min. The supernatant was removed, and the pellet was resuspended in buffer A (50 mM Tris, pH 7.5, 2 mM MgCl₂, 5 mM dithiothreitol, 10% glycerol). KCl was added dropwise to a final concentration of 400 mM. The homogenate was centrifuged at 10,000 × g for 10 min. The resultant nuclear extract was stored at −80 °C in aliquots.

RNA Affinity Chromatography—0.5 ml of avidin A beads (Vector Laboratories) were incubated with 36 μmol of biotinylated let-7 precursor hairpin RNA in 0.6 ml volume with buffer A (50 mM Tris, pH 7.5, 0.01% Nonidet P-40, 10% glycerol) at 4 °C for 8 h. Beads were washed with buffer A containing 1 M NaCl and then equilibrated with buffer A containing 50 mM NaCl. Nuclear extract was applied to the column and washed with buffer A containing 50 mM NaCl. The column was then eluted with buffer A containing a 50 mM stepwise 0.05 M-0.8 M NaCl gradient.

Preparation of Recombinant His-P68 RNA Helicase—Recombinant His-P68 was prepared as described previously (31). In short, His-P68 was induced in BL21 (DE3) cells with 0.1 mM isopropyl-1-thio-D-galactopyranoside at 37 °C for 6 h. Bacterial pellets were resuspended in lysis buffer (50 mM Tris, pH 8.0, 0.1 M NaCl, 0.5 mM EDTA, pH 8.0) and were lysed by the addition of lysozyme (0.2 mg/ml) and Triton X-100 (1%). The resulting supernatant was applied to a nickel-nitrotriacetic acid agarose column. The column was first washed with lysis buffer containing 20 mM imidazole followed by lysis buffer containing 20 mM imidazole and 0.15 mM NaCl. Recombinant His-P68 was then eluted with buffer containing 250 mM NaCl and 250 mM imidazole.

Preparation of Labeled MicroRNA Precursor Duplex—The let-7 guide strand was labeled using T4 polynucleotide kinase and [γ-32P]ATP (Amersham Biosciences). After phenol-chloroform extraction, it was annealed (65 °C for 5 min and then 37 °C for 25 min) to a 5-fold excess of let-7 passenger strand. The duplex was then gel-purified and stored in 50 mM Tris, pH 7.5, and 0.2 M potassium acetate.

Precursor Duplex-unwinding Assay—Reaction mixtures (0.02 ml) contained labeled let-7 precursor duplex (1 nM), 50 mM Tris, pH 7.5, 50 mM NaCl, 2 mM MgCl₂, 5 mM ATP, and nuclear extract or recombinant P68 RNA helicase as indicated.

FIGURE 1. Identification of a novel activity from human cells that unwinds the let-7 duplex. A, sequence and structure of the Let-7 microRNA duplex substrate. The microRNA or guide strand is the bottom strand. B, the duplex let-7 substrate and its single stranded derivative (produced by heat penetration) as analyzed by native gel electrophoresis. C, 32P-labeled let-7 duplex was incubated with increasing amounts of crude extract made from HeLa cells (amounts indicated). Reactions were deproteinized and analyzed for the presence of single-stranded RNA using native polyacrylamide gel electrophoresis. The asterisk indicates the addition of heat-inactivated extract. D, crude HeLa cell extract was fractionated using a Sephadex S-200 column. Fractions (2 μl) were assayed for unwinding activity, using 32P-labeled let-7 duplex. Molecular mass markers are: alcohol dehydrogenase (150 kDa), bovine serum albumin (68 kDa), and cytochrome c (12.4 kDa). E, 32P-labeled let-7 duplex was assayed for unwinding activity, using fraction 20 from the Sephadex S-200 column (2 μl) in the absence or presence of different nucleoside cofactors (as indicated). The asterisk indicates a reaction containing a heat-inactivated Sephadex fraction.
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RESULTS

The Identification of an ATP-dependent Activity That Can Unwind the let-7 MicroRNA Duplex—We elected to use the human let-7 duplex (Fig. 1A) as a model since let-7 is absolutely conserved between worms and humans and has been biochemically studied in many systems (12, 33–39). This choice therefore permits the ready comparison of any identified human protein co-factors with those identified in other organisms. However, one disadvantage of the human let-7 duplex is that a discernible level of single strand is produced merely on incubation at 37 °C. Thus, in all experiments, we included a negative comparison control (a heat-inactivated corresponding cellular fraction) so that we could clearly distinguish any cellular helicase activity from the enzyme-independent background. Fig. 1B shows that the let-7 duplex was unwound on incubation with nuclear extract made from HeLa cells. The extent of unwinding titrated with the amount of extract added to the reaction. To extend this observation and to establish the native molecular weight of the activity, we fractionated the extract using a Sephadex S-200 column. Fractions were collected and assayed for helicase activity. There was a minor high molecular weight species; however, the majority of the unwinding activity eluted in an inclusion volume consistent with a native molecular mass of 68 kDa (Fig. 1C).

Next, using this partially purified material, we investigated whether the unwinding activity required ATP. Little unwinding was seen in the absence of ATP, whereas the addition of ATP markedly stimulated the reaction and appeared to saturate at 5 mM. In addition, no unwinding activity was seen in reaction mixtures containing AMP-PNP, a non-hydrolyzable ATP analog (Fig. 1D). From these observations, we concluded that we have identified a novel ATP-dependent activity in HeLa cell extract that is capable of unwinding the let-7 microRNA duplex and that this activity has a native molecular mass of ~68 kDa.

P68 RNA Helicase Co-purifies with the MicroRNA Duplex-unwinding Activity—Our initial observations suggested that the activity that unwinds the let-7 duplex precursor might correspond to P68 RNA helicase. P68 RNA helicase is an ATP-dependent RNA-unwinding enzyme of 68 kDa that has been found to be a subunit of the Drosophila-processing complex (8, 30–32, 40). Accordingly, we elected to affinity-purify our unwinding activity and investigate whether it co-purified with P68 RNA helicase. We prepared an affinity column by immobilizing a biotinylated hairpin sequence containing the let-7 passenger and guide sequences (Fig. 2A) to avidin beads.

HeLa cell extract was applied, the column was washed with low salt, and bound protein was eluted using a step gradient from 0.05 to 0.8 M NaCl (Fig. 2B). A significant portion of the microRNA helicase activity was retained and eluted at 200–250 mM NaCl (Fig. 2C). Next, we assayed for the presence of P68 RNA helicase by Western blot and found that it was precisely coincident with the unwinding activity (Fig. 2D). Thus, we concluded that P68 was indeed a strong candidate for the microRNA-unwinding activity.
P68 RNA Helicase Is Sufficient to Unwind the let-7 MicroRNA Duplex—Next, we investigated whether recombinant P68 was sufficient to unwind the let-7 microRNA duplex, and if so, whether its properties resembled those displayed by the native activity. First, we investigated the structural features of the microRNA duplex that are necessary for unwinding by the native activity. We synthesized two mutant derivatives of the let-7 duplex. In the first mutant (mutant 1), nucleotide substitutions were made so that every nucleotide of the microRNA guide strand was annealed to the passenger. This mutant is a “bad” siRNA in which the 5’ end of the guide strand is annealed to the passenger strand. Previous studies have shown that the guide strand of such an siRNA will not be readily incorporated into a silencing complex (21, 22, 26, 41). This mutant was a very poor substrate; virtually no unwinding activity was noted even on incubation with saturating amounts of affinity-purified unwinding activity (Fig. 3, A and B).

Next, we synthesized a mutant that lacked the internal bulges of the microRNA duplex yet retains the unpaired structure of the 5’ end of the guide strand. This mutant (mutant 2) is analogous to a “good” siRNA in which the guide strand is readily incorporated into the silencing complex (41). Importantly, this mutant was also a very poor substrate and indicates that the unwinding activity recognized the internal bulges and therefore can distinguish between an siRNA duplex and a microRNA duplex. Indeed, the importance of the internal bulges was illustrated by the observation that a third mutant, in which the bulges were retained and the 5’ end of the guide was annealed to the passenger strand, exhibited significant unwinding activity.

Importantly, recombinant P68 RNA helicase also displayed a marked preference for a microRNA duplex, and the critical role of the internal bulges was similarly evident. Thus, we concluded that P68 RNA helicase is sufficient to unwind the let-7 duplex, and like the affinity-purified activity, it prefers a microRNA duplex to an siRNA duplex (Fig. 3, A and C).
Evidence that this suppression was exerted by let-7 was provided by the observation that this suppression was alleviated by AntagomiRs against let-7 but not by AntagomiRs against an irrelevant microRNA (Fig. 4A). Moreover, the suppressive activity of the element was attenuated by mutations that compromise the annealing of let-7 (Fig. 4B). In the following experiment, we have used the comparison of the luciferase activity between the wild type (WT) and mutant (MUT) let-7-response elements as a measure of let-7 activity. siRNA-mediated down-regulation of P68 RNA helicase, but not GAPDH, attenuated the activity of let-7 microRNA (Fig. 4B). Importantly, GAPDH was successfully down-regulated as shown by Western blot analysis using Vimentin as a loading control (Fig. 4C). Thus, we conclude that P68 RNA helicase is indeed required for microRNA activity and is likely required for the incorporation of the microRNA into the Argonaute2 complex.

**DISCUSSION**

The single-stranded small RNA-directed silencing of mRNA has been reconstituted with recombinant Argonaute2 (18). However, the precursor microRNA duplex-directed silencing of mRNA has not yet been reconstituted with the candidate recombinant proteins. Thus, it is likely that other factors remain to be discovered. In these studies, we have identified an ATP-dependent unwinding activity that specifically unwinds the let-7 microRNA duplex yet exhibits little activity on a derived siRNA duplex. This observation reinforces the current perception that the guide strands of siRNA and microRNA duplexes arrive in Argonaute2 complexes through different pathways (20, 22, 26). Indeed, it will be interesting to see whether RNA helicase A, which has been implicated in the unwinding of the siRNA duplex (27), is also capable of unwinding some microRNA duplexes.

Our size fractionation analysis and affinity purification studies suggested that a principal component of the unwinding activity corresponds to the P68 RNA helicase. P68 was originally identified in human cells due to its coincidental reactivity with a monoclonal antibody directed against SV40 large T antigen (40).
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P68 was originally believed to be an RNA helicase due to its homology to the DEAD box of eIF-4A, a well characterized RNA helicase (43, 44). When P68 was assayed for unwinding activity, it was found to be an ATP-dependent RNA helicase, which can unwind RNA duplexes in both 3′ to 5′ and 5′ to 3′ directions (30–32). The substrates unwound by P68 RNA helicase range in size from 22 to 175 nucleotides in length and contain overhangs of varied lengths ranging from 6 to 185 nucleotides long (30–32). Thus, our observations are consistent with the known properties of P68 RNA helicase.

P68 RNA helicase has been implicated in many cellular functions. In some cases, for example, in its perceived role as a transcriptional regulator, this function does not require helicase activity (45). In most cases, however, its touted role in mRNA splicing, rRNA processing, and mRNA decay requires the integrity of the helicase domain (46–48). Our studies here lead us to speculate that P68 RNA helicase might regulate the expression of many microRNAs with a consequent pleiotropic effect upon cellular function. It is possible that this function might accommodate many of the previously ascribed functions in RNA metabolism. In any event, the regulation of microRNA activity would be consistent with its well described role in cellular proliferation. However, it remains possible that P68 RNA helicase may only unwind particular subclasses of microRNA duplexes, and its role in a particular cell function may be peculiar to the miRNAs that are expressed in a given cell type.

Our contention that P68 RNA helicase plays a role in the unwinding of the let-7 microRNA duplex is strengthened by the previous observation that it is a subunit of the affinity-purified Drosha-processing complex (48). In addition, it has been recently demonstrated that mouse embryonic fibroblasts that lack P68 RNA helicase are compromised in their expression of Drosha-processing complex (48). In addition, it has been recently demonstrated that mouse embryonic fibroblasts that lack P68 RNA helicase are compromised in their expression of Drosha-processing complex (48). In addition, it has been recently demonstrated that mouse embryonic fibroblasts that lack P68 RNA helicase are compromised in their expression of Drosha-processing complex (48). In addition, it has been recently demonstrated that mouse embryonic fibroblasts that lack P68 RNA helicase are compromised in their expression of Drosha-processing complex (48). In addition, it has been recently demonstrated that mouse embryonic fibroblasts that lack P68 RNA helicase are compromised in their expression of Drosha-processing complex (48). In addition, it has been recently demonstrated that mouse embryonic fibroblasts that lack P68 RNA helicase are compromised in their expression of Drosha-processing complex (48). In addition, it has been recently demonstrated that mouse embryonic fibroblasts that lack P68 RNA helicase are https://doi.org/10.1074/jbc.M600000200/...
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