Small RNAs with Imperfect Match to Endogenous mRNA Repress Translation

IMPLICATIONS FOR OFF-TARGET ACTIVITY OF SMALL INHIBITORY RNA IN MAMMALIAN CELLS

Received for publication, July 2, 2003, and in revised form, August 19, 2003
Published, JBC Papers in Press, September 2, 2003, DOI 10.1074/jbc.M307089200

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A 21-base pair RNA duplex that perfectly matches an endogenous target mRNA selectively degrades the mRNA and suppresses gene expression in mammalian tissue culture cells. A single base mismatch with the target is believed to protect the mRNA from degradation, making this type of interference highly specific to the targeted gene. A short RNA with mismatches to a target sequence present in multiple copies in the 3′-untranslated region of an exogenously expressed gene can, however, silence it by translational repression. Here we report that a mismatched RNA, targeted to a single site in the coding sequence of an endogenous gene, can efficiently silence gene expression by repressing translation. The antisense strand of such a mismatched RNA requires a 5′-phosphate but not a 3′-hydroxyl group. G-U wobble base pairing is tolerated as a match for both RNA degradation and translation repression. Together, these findings suggest that a small inhibitory RNA duplex can suppress expression of off-target cellular proteins by RNA degradation or translation repression. Proper design of experimental small inhibitory RNAs or a search for targets of endogenous microRNAs must therefore take into account that these short RNAs can affect expression of cellular genes with as many as 3–4 base mismatches and additional G-U mismatches.

Small inhibitory RNAs (siRNAs)1 are small RNAs of ~21-nucleotide length that trigger the destruction of target mRNA with which they share complementarity (1). In vivo, they are generated by an RNase III enzyme, Dicer, which cleaves a precursor double-stranded RNA (dsRNA) into ~21-nucleotide sense, antisense, or, potentially, dsRNA fragments (2–5). The ~21-nucleotide siRNA is incorporated into an RNA-induced silencing complex (RISC) that mediates the cleavage of target mRNA. RNA-mediated silencing was first observed in petunia silencing complex (RISC) that mediates the cleavage of target

1 The work was supported by National Institutes of Health Grant R01 CA89406 (to A. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: siRNA, small inhibitory RNA; dsRNA, double-stranded RNA; RNAi, RNA interference; stRNA, small temporal RNA; UTR, untranslated region; miRNA, microRNA.
the targets of miRNA share only partial complementarity, very little is known about the targets or functions of most miRNAs. Transfection of siRNA complementary to an mRNA is a fast and convenient tool for studying loss of function of a target gene in mammalian cells. However, there are conflicting reports in literature about the specificity of the sequence match necessary to achieve gene silencing (28–30). Recent reports by at least two different groups have claimed high specificity of RNAi silencing by analyzing genome-wide expression profiling (28, 30). However, others have observed that partial complementarity between siRNA and mRNA leads to nonspecific changes in mRNA levels, raising an interesting debate on specificity of siRNA silencing (29).

A recent report showed that mismatched short RNAs can translationally silence a target mRNA with multiple targets in the 3′-UTR (35). Because the target mRNA was an artificial construct expressed from an exogenous promoter, it was not clear how these findings could apply to the conventional siRNA experiments in which the short RNA duplex is targeted to a single site in the coding region of an endogenous gene. We report here that a short RNA duplex similar to those used in siRNA experiments can function like an stRNA and silence an endogenous gene that carries only one natural target sequence partially complimentary to it. We also report that the target for this stRNA-like process is not limited to 3′-UTR and can include the coding sequence of a gene. Taken together, our results emphasize the possibility of off-target silencing by siRNAs used for an experimental or a therapeutic purpose.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfections, Protein, and RNA Analysis**—PC3 cells from the American Type Culture Collection (Manassas, VA) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics, whereas HeLa cells were maintained in Dulbecco’s modified Eagle’s medium. For dsRNA transfection, commercial RNA duplexes composed of 21-nucleotide sense and 21-nucleotide antisense strands were used, paired so as to have a two-nucleotide 3′-overhang (Dharmacon and Ambion). Cells were grown to 10%, even after three dsRNA transfections. The cells were then assayed for levels of targeted protein and mRNA. A nonspecific RNA duplex (GL2), complimentary to firefly luciferase (5′-AACGUAACGGGGAUACUCUGA-3′), was used as a negative control.

**RESULTS**

**Imperfect Base Pairing with the Target Causes siRNA to Silence the Gene in an stRNA-like Process**—We initially designed an siRNA (p21-2) against a target in the 3′-UTR of the endogenous p21 mRNA (Fig. 1A). Upon transfection of PC3 cells, we observed that when 3–4 mismatches are introduced into p21 or geminin siRNA, they can still silence the targeted gene, but by triggering translational repression instead of mRNA degradation. The 5′-phosphate group is required at the antisense strand for translational repression in human cells. The 5′- hydroxyl group is dispensable, eliminating any role of a primer extension-mediated amplification step in dsRNA-mediated translational repression silencing. The G-U wobble base pair is recognized like an authentic Watson-Crick base pair in antisense RNA oligonucleotide-mRNA duplex interaction. Base pairing at the center of duplex is more critical, stabilizing the antisense RNA oligonucleotide-mRNA duplex and enhancing the silencing.
cells, this perfectly base-paired p21 siRNA caused an ~85% reduction in protein level (Fig. 1B, quantitation in Fig. 1C).

Next, we incorporated mismatches in the siRNA to observe the effect of the mismatch on silencing. With 3–4 mismatches (p21-4, p21-10, and p21-11) we still observed a significant decrease in p21 protein levels. However, the mRNA was not degraded (Fig. 1, A–C). These results contrast to those observed by others, wherein a scrambled siRNA (the complementary siRNA with an inversion of a four-nucleotide fragment in the middle of sequence) does not cause any changes in levels of target protein (28). Therefore, three mismatches in a geminin siRNA targeted to the coding region converts it to a function like an stRNA. Translational repression could be observed with three (GEM3) but not with five or eleven (GEM8, 7) mismatches (Fig. 2, A–C). Therefore stRNA-like silencing occurs on other endogenous genes and is not limited to the 3′-UTR.

Because detection of protein levels by enhanced chemiluminescence immunoblotting can be non-linear, we loaded varying amounts of cell lysate for immunoblotting to carefully quantify the decrease in protein levels. Around 4-fold more cell lysate had to be loaded from GEM3-transfected cells as compared with control cells to detect equivalent geminin levels (Fig. 2D, quantitation in Fig. 2E). This decrease to ~25% of endogenous protein levels was comparable with the decrease seen with perfectly matched siRNA.

**stRNA-like Silencing Can Be Observed Even When Targeted to the Coding Sequence of a Gene**—We next asked whether the stRNA-like silencing occurs in other genes and when targeted to the coding region of the mRNA. First, we designed a perfectly base-paired siRNA targeting the coding sequence of geminin mRNA (GEM2) (Fig. 2A). GEM2 siRNA reduces the protein and mRNA levels (Fig. 2B, quantitation in Fig. 2C). We then steadily incorporated mismatches within the siRNA. When three mismatches were incorporated with respect to the target (GEM3), there was significant decrease in protein but not in mRNA levels. Therefore, three mismatches in a geminin siRNA targeted to the coding region converts it to a function like an stRNA. Translational repression could be observed with three (GEM3) but not with five or eleven (GEM8, 7) mismatches (Fig. 2, A–C). Therefore stRNA-like silencing occurs on other endogenous genes and is not limited to the 3′-UTR.

**G-U Wobble Base Pairing in Antisense RNA Oligonucleotide-mRNA Target Interaction**—The RNA secondary structure permits G-U wobble base pairs that have a thermodynamic stability comparable with Watson-Crick base pairs (37). If wobble base pairing is tolerated in the antisense RNA-target duplex, it would expand the range of potential targets for the
same siRNA. To test whether wobble base pairing is tolerated in RNA-mediated silencing, we used a perfectly matched p21 siRNA that causes mRNA degradation (p21-2) (Fig. 3, A and B). Three nucleotides were replaced such that the siRNA would form three G/U wobble base pairs with the target (p21-3). The protein silencing and the mRNA degradation were unaltered. On the other hand, replacing the same three nucleotides such that there are "real" mismatches with the target (p21-4) still silences protein expression, but this is via an stRNA-like pathway (decrease in protein without change in mRNA levels; Fig. 3, A and B, with quantitation in Fig. 3C). Therefore the three G-U wobble base pairs were recognized as authentic base pairs, and replacing them with non-wobble base pairs (which are recognized as mismatches) leads to stRNA-type activity.

We next investigated whether wobble base pairs can expand the spectrum of stRNA-like silencing. It has been reported that G-U base pairing is recognized as a mismatch in micro-RNAs leading to disruption of silencing (34). GEM6 is a geminin dsRNA duplex that silences endogenous geminin by translational repression (Fig. 3, D and E, with quantitation in Fig. 3F). This dsRNA duplex has three real mismatches and two G-U
wobble base pairings with respect to the target. It does not degrade the target mRNA but silences the target protein expression. When the two G-U base pairs were changed to non-wobble mismatches (five real mismatches; GEM8), there was a significant reduction in silencing (Fig. 3, D–F). Therefore, as in mRNA degradation, wobble base pairing functions as authentic base pairing in translational repression, expanding the gamut of potential off-target genes that may be silenced by siRNA-like processes.

Base Pairing in the Center of the Target Region Is Critical for RNA-mediated Silencing—We tested whether mismatches at the ends are better tolerated than mismatches at the center of the RNA oligonucleotide–mRNA duplex. The antisense strand of a transfected dsRNA duplex is believed to be the functional strand in gene silencing. A perfectly base-paired RNA (p21-1) targeting the coding sequence of the p21 gene silences p21 efficiently (Fig. 4, A and B). When base pairing was disrupted at the 3′-end of the complementary region, we still observed effective silencing (p21-1g). However, when mismatches were introduced at the center of the base-pairing region (position 7–13 relative to the 5′-end of the antisense strand), no silencing was observed (p21-1h). Therefore, mismatches were better tolerated at the end of the duplex than in the middle. It is also of interest that a two-base pair mismatch in p21-1h abrogated silencing, whereas mismatches of three or four bases in p21-4, p21-10, and p21-11 still permitted silencing. Therefore, it is not simply the number of mismatches but also the context or nature of the mismatch that determines whether an oligonucleotide functions like an siRNA.

Translational Repression Can Be Induced by Single-stranded Antisense RNA—We next determined whether some of the rules by which siRNAs function in mammalian cells were also applicable to gene silencing via translational repression. Single-stranded antisense RNA has been shown to be effective as an siRNA (38). We therefore asked whether the single-stranded antisense RNA can induce silencing by translational repression. Consistent with published results, antisense single strand siRNA was as effective as double-stranded siRNA (GEM2) for silencing geminin expression (Fig. 4C, with quantitation in Fig. 4D). Transfecting the antisense strand of GEM3 also produced effective silencing, just like double-stranded GEM3 (Fig. 4, C and D). The sense strand of GEM3 did not change geminin levels. Therefore, a single-stranded antisense RNA that can imperfectly pair with the target mRNA can induce translational repression.

5′-Phosphate Is Required for stRNA-like Silencing in HeLa Cells—The antisense strand of siRNA needs a 5′-phosphate group for activity (38, 39). For the studies above, we used 5′-hydroxylated dsRNA, which is believed to be rapidly phosphorylated in vivo by endogenous kinases. We investigated the requirement of a 5′-phosphate group on the antisense strand of an RNA duplex for translational repression. For this we used an RNA duplex blocked at the 5′-end by an amino group with a three-carbon linker (as/5′-N3; Fig. 5A). These modified RNA duplexes lack the 5′-hydroxyl group and cannot be phosphorylated by kinases in vivo. We observed that these modified RNA duplexes did not silence gene expression (Fig. 5B, with quantitation in Fig. 5C). Similar to siRNA, the 5′-phosphate group on the antisense strand is essential for dsRNA to enter into the “stRNA-like silencing complex” to cause translational repression (38, 39).

The 3′-hydroxyl group in the antisense strand of siRNA, which is essential in C. elegans, is dispensable for silencing in mammalian cells. We therefore tested whether stRNA-like silencing requires the 3′-hydroxyl group as a primer for RNA synthesis following annealing to the target RNA-template. The 3′-hydroxyl group of the antisense strand was blocked with 3-aminopropyl phosphoester (3′-amino modifier) so that it could no longer be used as primer for extension (as/3′-N3–3′; Fig. 5A). There was no difference in silencing with 3′-modified oligonucleotides, suggesting that RNA-mediated translational repression does not involve amplification of RNA by primer extension from the 3′-hydroxyl end (Fig. 5, B and C).
Mismatches Are Tolerated at the 3'-End but Not the 5'-End of the Antisense Strand—In light of the observation that modifications at the 5'-end are not tolerated in RNA-mediated silencing, we tested whether mismatches at the 5'-end are tolerated. Unlike mismatches at the 3'-end, incorporating mismatches at the 5'-end of an antisense strand abolished silencing (Fig. 5, D and E, with quantitation in Fig. 5F). Therefore, for the p21 mRNA, incorporating >3–4 mismatches in the center of the target region or mismatches at the 5'-end of the antisense strand limits RNA-mediated silencing.

**DISCUSSION**

Different workers have reported varying degrees of tolerance for mismatches in siRNA-mediated silencing (13, 31–33). Although some report that a bulge or a 2–3 nucleotide mismatch is permissible in siRNA-mediated silencing, others observe that even a single base pair mismatch completely eliminates silencing (35, 40). Here, we have systematically increased the mismatches between the short RNA and target RNA. We observe that as mismatches increase, an siRNA begins to function like an stRNA. Therefore, we note that a 21-nucleotide double-stranded RNA that shares only partial complementarity with the target mRNA is still competent to cause gene silencing via translational repression. We also note that the antisense strand alone, but not the sense strand of the same duplex, can induce silencing.

Adaptation of an siRNA to function like an stRNA implies that complementarity is a critical factor defining the mode of...
action of these small RNA molecules. Hutvagner and Zamore demonstrated that in human cell extracts a natural stRNA, let-7, can function as siRNA with a perfectly base-paired target (41). Zeng et al. designed an miRNA that inhibited the translation of a target mRNA with which it shared partial complementarity (40). The target was an exogenously expressed mRNA in which four copies of the partial complementary sequence were inserted at the 3′-UTR. Similarly, Doench et al. reported that siRNA can function like an stRNA if it contains a central bulge with respect to the target (35). These workers again used an exogenously expressed mRNA in which four consecutive binding sites were engineered in the 3′-UTR. Here, we report that an RNA duplex can silence an endogenous gene with which it has a single binding site with up to 3–4 mismatches. This was demonstrated using two genes (p21 and geminin) in two different cell lines (PC3 and HeLa).

Various reports in literature suggest that the target of an stRNA has to be the 3′-UTR (22–24). However, recently Kawasak and Taira have reported a natural micro-RNA, miR-23, that is partially complementary to the coding region and stop codon of the target mRNA (42). We now show that a mismatched RNA designed to target the coding sequence of the gene can function like an stRNA.

The perfectly base-paired siRNA (GEM2) silences the gene by mediating degradation of geminin mRNA. Although the silencing was complete, with undetectable protein levels, low levels of mRNA could still be detected. Therefore, it is possible that gene silencing mediated even by a perfectly base-paired siRNA like GEM2 involved a component of translational repression. It is likely that this serves as an additional or redundant mechanism (in case the mRNA target degradation is unsuccessful for some reason) to effect complete silencing.

We have identified the characteristics of dsRNA important for it to function like an stRNA. The 5′-phosphate but not the 3′-hydroxyl group of the antisense strand is required for translational repression in human cells. The requirement of the 5′-phosphate for stRNA-like activity is similar to that observed in siRNA (38, 39). On the other hand, it is clear that, with siRNA in human cells, amplification of an RNA duplex by primer extension from the 3′-hydroxyl end of the antisense strand is not required for translational repression in human cells.

Can we definitely predict the efficacy of dsRNA-mediated silencing based on the number of mismatches with the target? Two mismatches with the p21 mRNA (p21-h) abolishes silencing, whereas the same number of mismatches with geminin mRNA (GEM3) permits silencing. Therefore, it is not the absolute number of mismatches but probably the overall stability of the antisense RNA oligonucleotide-mRNA duplex that determines the success of a silencing event. This study suggests that additional experiments are necessary to explicitly predict the efficacy of silencing of different targets and sequences on the basis of number of mismatches. The secondary structure of the mRNA target and the sequence composition of the short RNA likely contribute to the stability of the duplex. Therefore, additional targets and sequences should be tested systematically to define broad rules for dsRNA-mediated silencing.

Is there any advantage for a cell to allow gene silencing by short RNA that has only partial complementarity? It has been suggested that si/stRNAs evolved as a defense mechanism against viruses and other intracellular pathogens. Because mismatched oligonucleotides can suppress protein expression, pathogens with a few nucleotide changes at the target site will still be inhibited by RNA duplexes. In addition, a single short RNA to a relatively conserved region might inhibit multiple cellular genes in the same family. It should also be noted that, because short RNA duplexes with mismatches and wobble base pairs can silence endogenous genes, programs searching for targets of siRNA should allow for such mismatches and wobble base pairs.

There are numerous reports in the literature describing the phenotypes following inactivation of various genes by siRNA duplexes (43). This study cautions that the silencing phenotypes observed by small RNA-mediated silencing should be interpreted conservatively, because there are now at least two additional mechanisms by which the target range is extended, namely mismatches and GU wobble base pairs. Recently Jackson et al. have shown that partial complementarity between siRNA and mRNA leads to nonspecific changes in mRNA levels (29). We show here that partial complementarity can also lead to translational repression, thereby increasing the range of nonspecific effects. To eliminate off-target silencing effects, a simple precaution is to use two or more separate duplexes targeting different regions of an mRNA to look for a reproducible phenotype.

Acknowledgments—We thank members of Dutta laboratory for valuable discussions. Kazutaka Murata is acknowledged for technical help and for the insight on wobble base pairs, and Chinweike Ukoma is thanked for the p21-1 and p21-2 siRNAs.

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