mRNA translation is not a prerequisite for small interfering RNA-mediated mRNA cleavage

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Abstract RNA interference constitutes a major means of eliminating mRNAs, yet how the small interfering RNAs (siRNA) within the RNA-induced silencing complex (RISC) finds its homologous target in the cell remains unknown. An attractive hypothesis is that RNA interference is linked to translation which allows RISC ready access to every translated mRNA. To test whether translation could direct siRNAs to mRNAs, chemical and biological inhibitors of translation and their effects on mRNA cleavage were tested. Our results show that mRNA degradation by siRNAs is not dependent on mRNA translation.

Key words RNAi, siRNA, translation, RISC, gene regulation

Introduction

In a diverse group of organisms ranging from Caenorhabditis elegans to Homo sapiens, the introduction of double-stranded RNAs elicits an RNA interference response which inhibits gene expression in a sequence-specific manner (Fire et al., 1998; Shi, 2003). Such inhibitory RNAs have become highly useful tools for generating loss of function organisms or cells leading to the elucidation of critical signaling pathways. Yet, much remains to be elucidated regarding the mechanisms by which they act. Double-stranded RNAs are cleaved by Dicer, an RNASE III enzyme, which yields small interfering RNAs (siRNAs) of 19–21 bp (Hannon, 2002). These siRNAs are then bound by a protein complex designated as RNA-induced silencing complex (RISC) which unwinds the double-stranded siRNA. Together with the RISC complex, this single-stranded siRNA then associates with its complementary mRNA. Once the RISC complex reaches its target mRNA, an endonuclease, Arogonaute 2, cleaves that message resulting in its degradation and a specific loss of gene expression (Liu et al., 2004; Meister and Tuschl, 2004). Currently, the mechanism by which the siRNA within the RISC complex associates with its target mRNA remains unknown.

An attractive hypothesis as to how the siRNA within the RISC encounters its target mRNA is by association with the translation machinery. This would allow the RNA interference machinery to have ready access to all transcripts. Although appealing, compelling evidence that RNA interference is coupled to translation is lacking and the question is still an active topic of debate (Wang and Carmichael, 2004). The strongest evidence in support of a link between RNA interference and translation derives from experiments in Drosophila oocytes in which untranslated, but not translated, mRNAs were spared from degradation when targeted by RNA interference (Kennerdell et al., 2004). Interpretation of these data are subject to controversy, however, as the state of the untranslated mRNAs in oocytes is not entirely clear. For example, the mRNA could be bound to proteins that prevent access to the translational machinery as well as the RNA interference components, which would be coincidental but not causal. Other evidence in support of a link between RNA interference and translation derives from experiments in which the RNA interference machinery cofractionates with the 80S...
ribosomal complex in Drosophila extracts (Pham et al., 2004). Many of the components are shared by the RISC and microRNA-containing complex (miRNP). As miRNPs are known to block translation and are associated with ribosomes, the RISC complex could operate in a similar manner (Mourelatos et al., 2002).

Evidence that translation is not a prerequisite for RNA interference derives primarily from in vitro mRNA assays using cell lysates in which siRNAs directed against radiolabeled target mRNAs were cleaved despite the presence of translational inhibitors (Zamore et al., 2000). There are several caveats to these experiments. First, chemical inhibitors can have pleotropic effects, especially in cell lysates. Second, the inhibitors used in these studies were translation elongation inhibitors which does not rule out the necessity for translational initiation in mRNA cleavage. Third, an in vitro system may not accurately reflect the in vivo situation.

To determine definitively whether translation of an mRNA is a prerequisite of the RNA interference-mediated cleavage of that mRNA, we utilized two approaches. First, chemical inhibitors of mRNA translation were used in vivo in the context of intact cells, not cell lysates. Second, a well characterized system for regulating translation based on a biological regulator, iron, was used. Iron is well known to control the translation of ferritin 5′-untranslated region (5′UTR) linked reporter gene constructs in a dose-dependent manner (Iwai et al., 1995; Macchi et al., 2003). In either case, if the mechanism of action of RNA interference were coupled to translation, then only translated transcripts would be susceptible to RNA interference-mediated cleavage. The ferritin construct is particularly advantageous as a test system because it avoids nonspecific effects on cell physiology often associated with global inhibitors of translation, alters the translation of particular mRNAs, and acts via a mechanism that has been well documented to be the first step in the translation pathway. In this report, similar to results by Giu et al., we demonstrate that mRNA translation and cleavage via siRNA can clearly be dissociated and are not interdependent (Giu et al., 2005).

**Methods**

**Constructs**

The iron-regulated construct (IRC) construct was generated by PCR amplifying the promoter and IRE containing 5′UTR from the rat ferritin gene using the following primers (ASEI Ferritin: CCCATTATAGCCCCAAAGGATGCGCTTCAC and AgeI Ferritin: CCAACCCGTTGATGGCGCCTGGGAGGCG) The plasmid containing the Rat ferritin promoter and 5′UTR was a kind gift from Paolo Macchi (Tubingen, Germany). The rat ferritin promoter and 5′UTR was cloned into the ASEXAGEI of the pEYFP-N1 vector (Clontech, Palo Alto, CA). The rat ferritin promoter and 5′UTR fused to enhanced yellow fluorescent protein (eYFP) was PCR amplified using the following primers (EcoRI rat ferritin: CGGAATTCCAGCCCGAGGATGCGCTTCAC and Xho SV40: CCCCTCGAGGATGAGTTTGGACAAACCACAAC-TAG) and cloned into the EcoRI/Xho site of the retroviral vector pSuperior.Retro.Neo (Oligoengine, Seattle, WA) to generate the IRC construct. The retroviral short hairpin RNA (shRNA) constructs targeting eYFP and murine Oct-3/4 were generated as previously described (Sen et al., 2004). Two shRNA constructs targeting eYFP were used. One targeting the sequence 241–261 and another targeting 489–507 starting from the first start codon. Data was shown for the YSI retroviral construct which targets sequence 489–507.

**Cell culture**

Human embryonic kidney (HEK)-293 cells stably expressing the ecotropic receptor was a kind gift from Roland Wolkowicz (Stanford, CA). Cells were maintained in DMEM and 10% FCS.

**Translational inhibitors**

Cyclohexamide and clotrimazole were purchased from Sigma (St. Louis, MO) and used at 5 and 17.5 μg/ml, respectively. 293-retroviral construct eYFP (RY) and 293-RY-YSI cells were treated for 2, 4, or 6 hr with either drug and harvested for total RNA.

**Iron chelators and supplements**

The iron donor, ferric ammonium iron citrate, and iron chelator desferoxamine mesylate were purchased from Sigma and used at 16 and 3.3 μg/ml, respectively. Cells were treated with the drugs for 3 days and analyzed by flow cytometry and harvested for total RNA.

**RNA isolation and semi-quantitative RT-PCR**

Total RNA was extracted from HEK-293 cells using the RNasy mini kit (Qiagen, Hiden, Germany). One microgram of total RNA was reverse transcribed using the 1st Strand cDNA Synthesis Kit for RT-PCR (Roche, Mannheim, Germany). One microlitre of cDNA was used for amplification using the Titanium Taq PCR kit from Clontech. The PCR cycle for all reactions consisted of 94°C/1 min, 63°C/1 min, and 72°C/1 min, with the number of cycles dependent on each gene. Mouse β-actin primers were purchased from Stratagene (La Jolla, CA), eYFP primers that spanned the cleavage site of both eYFP shRNA retroviral constructs were used. The eYFP primers were as follows (eYFP for: GAAGTT-CATCTGCACCATCGCAACAG, eYFP rev: GGTGCTGAGTGGTTGTCG).

**Stable cell line production**

Ectropic phoenix cells (gift from Garry Nolan) were transfected with 1.6 μg of each retroviral expression construct. Transfections were carried out in 12 well plates using Fugene 6 (Roche) according to manufacturers instructions. Viral supernatants were collected 48 hr post transfection and polybrene added (5 μg/ml). These supernatants were placed on target cells and centrifuged for 30 min at 2,000 x g. 293-RY and 293-IRC Cells were infected three times and selected with neomycin (1 mg/ml). After the generation of 293-IRC cells, they were subjected to the same infection protocol using the YSI retroviral or OSI construct. 293-IRC-YSI or 293-IRC-OSI cells were selected in puromycin (1 μg/ml) 1 day after the last infection.
**Results**

Drug-induced translational inhibition

To determine whether translation is needed for RNA interference activity, we assessed mRNA cleavage in the presence of translational inhibitors in intact cells. For this purpose, cells were generated that expressed the eYFP in the presence of siRNAs directed against that reporter gene. If RNA interference activity were coupled to translation, the cell should exhibit greater cleavage in the absence of translational inhibitors. This *in vivo* cleavage assay is advantageous over previously used *in vitro* cleavage assays using cell extracts (Zamore et al., 2000), as all constituents of the RNA interference pathway remain present at physiological levels and in their appropriate intracellular compartments.

The constructs and cell lines used in the *in vivo* cleavage assays were derived as follows: first, HEK-293 cells were transduced with a retroviral construct driving the expression of eYFP under the control of the E1FA promoter, designated as RY in Fig. 1A. A phosphoglycerate kinase (PGK) promoter driving expression of the neomycin resistance gene within the same construct (not shown) enabled HEK-293 cells in which the RY construct was integrated to be selected for 5 days in the presence of G418 (designated 293-RY cells). After selection, one half of the 293-RY cells were stably transduced with a retroviral construct encoding a shRNA that targeted the eYFP reporter gene. The shRNA in the construct (YSI) targeted sequence 489–510 of the eYFP transcript and was generated by the REGS method which we previously developed (Sen et al., 2004). 293-RY cells stably transduced with the YSI construct were selected in puromycin for 5 days (designated 293-RY-YSI cells), as the retroviral vector included a PGK promoter driving expression of the puromycin resistance gene (not shown).

For assays of translation, both cell types described above were analyzed for expression of eYFP by flow cytometry. Cells containing the shRNA construct against eYFP exhibited a 10-fold knock-down of the mRNA, evident as a reduction of the eYFP protein (Fig. 2A). To examine the effects on 293-RY-YSI cells by translational inhibitors, cells were exposed either to cyclohexamide or clotrimazole. Cyclohexamide blocks translation elongation by preventing translocation of the ribosome along the mRNA (Carrasco et al., 1976). Clotrimazole causes depletion of intracellular calcium resulting in the activation of the PKR response and phosphorylation of eIF2α, blocking the initiation of translation (Aktas et al., 1998). Both inhibitors were confirmed to be functional at the concentration used in these particular cells by flow cytometry analyses of a destabilized eGFP (data not shown). Cells were treated for 2, 4, or 6 hr with the inhibitors and semiquantitative RT-PCR was performed to determine the mRNA levels for eYFP. If translation were necessary for siRNA-mediated cleavage, then blocking translation using these small molecules should prevent cleavage of the eYFP mRNA. Figure 2B shows that this is not the case. At each time point, treatment with either clotrimazole or cyclohexamide did not block siRNA-mediated cleavage of the eYFP mRNA. Clotrimazole treatment did not enhance or decrease the siRNA-mediated cleavage of the eYFP mRNA. The levels of the housekeeping gene β-actin were consistent throughout the treatment with either translational inhibitor (Fig. 2B, lower panel).

These results indicate that translation is not a prerequisite for RNA interference, but the experiments are not definitive. Such global inhibitors of translation are
generally toxic, have pleiotropic effects and resulted in apoptosis of 293-RY-YSI cells within 24 hr.

Iron-regulated inhibition of translation

To determine definitively whether siRNA-mediated cleavage of a target mRNA occurs in the absence of translation, we used a well characterized system for controlling translation using iron. In order to control the translation of a specific mRNA, we linked the 5′UTR of the ferritin gene to a reporter gene (eYFP) designated as the IRC. The ferritin 5′UTR contains Iron Response Elements (IREs) that control translation (Hentze et al., 2004; Goosen et al., 1990). In the presence of iron, translation is initiated, whereas in the absence of iron, translation ceases. Moreover, the effects on translation are graded and dose-dependent. The IRE forms a stem loop structure to which iron-binding proteins 1 and 2 (IRP1 and 2) complex (Fig. 1B). Once

Fig. 2 Translational inhibitors, cyclohexamide and clotrimazole, do not block RNA interference-mediated cleavage of target mRNA. (A) Flow cytometry of human embryonic kidney (HEK)-293 cells stably expressing enhanced yellow fluorescent protein (eYFP) (293-RY) and the resultant knock-down of eYFP expression when infected with YSI (293-RY-YSI). (B) 293-RY-YSI cells were treated with cyclohexamide or clotrimazole for 2, 4, or 6 hr. Cells were harvested and total RNA extracted. Semi-quantitative RT-PCR was performed to determine eYFP mRNA cleavage in the presence of translational inhibitors. 21, 24, and 27 cycles of RT-PCR were used to determine mRNA levels for eYFP. Twenty-seven cycles of RT-PCR is shown on the blot but results are similar for all cycles (Fig. 2B, upper panel). Experiments were performed two independent times. β-actin was used as an internal control using 17 and 19 RT-PCR cycles. Nineteen cycles are shown on the blot (Fig. 2B, lower panel).

Fig. 3 Translation of enhanced yellow fluorescent protein (eYFP) can be controlled by iron. (A) Flow cytometry was performed on 293 cells transduced with the eYFP construct that can be regulated by iron (iron-regulated construct, IRC). Translation of eYFP can be controlled in 293-IRC cells treated with the iron chelator (green), desferrioxamine mesylate, treated with regular medium (blue), or treated with the iron supplement (yellow), ferric ammonium iron citrate for 3 days. (B) 293-IRC cells were infected with the YSI retroviral construct targeting eYFP and analyzed for the knock-down of eYFP expression using flow cytometry.
bound, IRP1 and 2 prevent translation by blocking access and assembly of the small ribosomal subunit to the mRNA in the first step of translation (Muckenthaler et al., 1998). In the presence of iron, the IRPs bind to iron and lose affinity for the IRE allowing translation to occur. (Fig. 1B) When the IRE is cloned upstream of a reporter mRNA such as eYFP, translation of that particular mRNA can be controlled by the addition or removal of iron (Macchi et al., 2003; Goosen and Hentze, 1992). The use of this reporter mRNA linked to IREs in conjunction with siRNAs that target the mRNA allowed a definitive determination of whether RNA interference is coupled to translation in intact cells.

The cell system designed to determine whether translation could be controlled by iron without affecting transcription used HEK-293 cells transduced with retroviruses encoding IRC. Cells were selected in the presence of neomycin and assayed for their ability to control the translation of eYFP in the presence or absence of iron by flow cytometry. In the presence of the iron supplement ferric ammonium citrate, robust translation of eYFP occurred, as shown in the yellow histogram (Fig. 3A). By contrast, in the presence of the iron chelator, desferrioxamine mesylate, translation was inhibited (green histogram). Thus, a 10-fold difference in translation was detected by flow cytometry of cells in high- and low-iron conditions (Fig. 3A, compare yellow and green histograms). Cells grown in standard HEK-293 cell culture medium exhibited an intermediate level of translation.

Experiments were then designed to determine whether the inhibition of translation affected the potential of siRNAs complementary to the mRNA (IRC) to cleave its target. The 293-IRC cells were transduced with the YSI shRNA retroviral construct that targets the eYFP encoding mRNA (293-IRC-YSI cells) or the control OSI shRNA retroviral construct that targets the murine Oct-3/4 gene (293-IRC-OSI). The 293-IRC-YSI cells exhibited a 10-fold knock-down or decline in eYFP expression, a translational inhibition comparable with that observed when 293-IRC cells were treated with the iron chelator, desferrioxamine mesylate (see black and green profiles, Fig. 3B).

Translation was further manipulated by iron concentration in order to examine its effects on the potential of siRNA to cleave its target mRNA. The 293-IRC-YSI and 293-IRC-OSI cells were cultured in low- or high-iron conditions to inhibit or enhance translation. An increase in translation of eYFP was evident in the presence of high iron, while a decrease in translation occurred in low iron (Fig. 4A) for both cell types. The 293-IRC-YSI cells on average exhibited a log decrease in eYFP protein levels than 293-IRC-OSI cells since they contain an shRNA construct targeting the eYFP gene. To determine whether the increase or decrease in eYFP protein levels was because of increased or decreased
increased translation and not an increase or decrease in transcription, mRNA was harvested from the control 293-IRC-OSI cells and semi-quantitative RT-PCR was used to determine the mRNA levels in all iron conditions. As shown in Fig. 4B, the eYFP mRNA levels remained constant in the presence (Fe) or absence of iron (C) in 293-IRC-OSI cells. The mRNA levels for the housekeeping gene β-actin also remained constant over time (Fig. 4C), indicating that iron levels do not change the abundance of transcripts encoded by other genes. Thus, the changes in eYFP resulted from changes in translation because of iron concentration, not changes in transcript levels.

To determine conclusively whether translation is needed for mRNA cleavage, mRNA was isolated from 293-IRC-YSI cells that had been treated for 3 days in the presence of high iron, medium iron, or low iron. Semi-quantitative RT-PCR using primers that span the mRNA cleavage site was performed to determine if there was enhanced or inhibited cleavage. As shown in Fig. 4B, there was no difference in the mRNA levels for 293-IRC-YSI cells in the presence or absence of translation. The mRNA levels were the same for all PCR cycles tested and the levels of a control housekeeping gene Actin also remained unchanged (Fig. 4C). These results were not because of the region of eYFP targeted, as similar results were obtained using an YSI shRNA construct generated by REGS that targets a different sequence, 245–266, of the eYFP mRNA. These data demonstrate that active translation of mRNA is not necessary for RNA interference mediated mRNA cleavage.

Conclusions

The idea that RNA interference is intimately linked to translation is attractive. This mechanism of regulation would allow for the scanning of every translated mRNA by the RNA interference machinery. Published reports suggest that a connection exists (Kennerdell et al., 2002; Pham et al., 2004; Wang and Carmichael, 2004), but definitive proof is lacking. Our data clearly demonstrate that active translation is not essential for siRNA-mediated cleavage of mRNA. We used two different systems to address this question. In one system, we assayed for cleavage of mRNA following treatment with translational initiation and elongation inhibitors in intact cells. These inhibitors did not block the degradation of the reporter mRNA in the presence of targeting siRNAs. However, translational inhibitors are not optimal as they globally inhibit translation and can result in nonspecific effects. Thus, in order to rule out a dependence of RNA interference to translation we turned to an elegant and well-characterized system of iron-regulated translation. This second system offers many advantages. These include the fact that translation of a specific mRNA can be controlled at the level of initiation thereby blocking all subsequent steps in the pathway. In addition, control of translation is regulated by the concentration of a single molecule, iron. Because of the unique features of this system, we can clearly conclude that although co-regulation of the two processes would seem advantageous, they clearly are not.

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