

The Ribosomal Protein RACK1 is involved in the microRNA pathway of both *C. elegans* and humans

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Running Title: RACK1 function in miRNA-mediated gene regulation

Abstract

Despite the importance of microRNAs in gene regulation, it is still unclear how the microRNA-Argonaute complex (or miRISC) can regulate the translation of their targets in such diverse ways. Here we demonstrate a direct interaction between the miRISC and the ribosome by showing that a constituent of the eukaryotic 40S subunit, RACK1, is important for miRNA-mediated gene regulation in animals. *In vivo* studies demonstrate that RACK1 interacts with components of the miRISC in nematodes and mammals. In both systems, the alteration of RACK1 expression alters miRNA function and impairs the association of the miRNA complex to the translating ribosomes. Our data suggest that RACK1 can contribute to recruit the miRISC to the site of translation and support a post-initiation mode of miRNA-mediated gene repression.

Keywords: ALG-1/hAGO2 /RACK1/miRISC recruitment/miRNA

Introduction

Initially discovered in *C. elegans*, microRNAs (miRNAs) have emerged as common cellular components with conserved functions in both animals and plants. In all species miRNAs associate with Argonaute proteins to form the core effector complex, known as the miRNA-Induced Silencing Complex or miRISC, which is capable of altering protein synthesis and/or inducing mRNA destabilization. In animals, there are several members of the Argonaute gene family and all are essential for small RNA mediated silencing pathways (reviewed in Hutvagner & Simard, 2008).

In recent years, it has been reported that the miRISC can influence translation in distinct ways (reviewed in Filipowicz et al, 2008). Interestingly, it has been recently observed in *Drosophila* that the identity of the Argonaute protein associated with miRNA can dictate which mechanism will lead to translational inhibition (Iwasaki et al, 2009). This new observation suggests that the Argonaute constituent of the miRISC as well as its interacting proteins will contribute to regulating protein expression by different mechanisms.

In this study, we identify the **R**eceptor for **A**ctivated **C**-**K**inase (RACK1) as an interactor of both *C. elegans* and human miRISC and demonstrate the importance of RACK1 for miRNA-mediated gene silencing in both systems. We observe that the loss of RACK1 affects the association of miRNA and Argonaute with translating ribosomes, suggesting that this component of the 40S ribosomal subunit can mediate the recruitment of the miRISC to the active site of translation.

Results and Discussion

RACK1 interacts with components of the miRISC of C. elegans

To gain a better insight into the miRNA pathway, we carried out a two-hybrid screen to identify proteins that interact with ALG-1, one of the two Argonaute proteins essential for miRNA-mediated gene regulation in *C. elegans* (Grishok et al, 2001). Among the proteins interacting with ALG-1, we identified K04D7.1, the ortholog of the mammalian protein RACK1 (Fig S1). Recently, ceRACK-1 has also been identified as a constituent of the ALG-1 complex by mass spectrometry (Chan & Slack, 2009). To confirm the relevance of this interaction, we generated tagged recombinant ALG-1 and ceRACK-1 proteins and performed GST pulldown assays. When compared to beads coupled to GST protein, we observed that GST tagged ALG-1 efficiently pulled down His-ceRACK-1 (Fig 1A). These data suggest that ceRACK-1 is *a bona fide* interactor of ALG-1 in *C. elegans*.

To address whether this interaction can occur *in vivo*, we generated a transgenic worm expressing GFP::ALG-1 and HA::ceRACK-1 to overcome the lack of specific antibodies. Using a whole worm lysate made from the transgenic *C. elegans*, we immunoprecipitated GFP::ALG-1 with HA::ceRACK-1 and conversely we detected HA::ceRACK-1 in the GFP::ALG-1 pulldown (Fig S2B). To monitor whether ceRACK-1 can also interact with miRNAs, we used a 2'-*O*-methylated RNA affinity matrix to trap sequence-specific small RNA complexes that are not bound to target mRNAs (Hutvagner et al, 2004; Yigit et al, 2006). Using this method, we detected both ALG-1 and ceRACK-1 associated with *let-7* and *lin-4* miRNAs (Fig 1B). To determine if the interaction between ceRACK-1 and the

miRISC reflects only a general interaction with the ribosomes, we generated transgenic animals expressing a HA-tagged 40S ribosomal protein. We observed that while the RNase treatment nearly abolished the interaction between the *let-7* miRNA complex and the 40S subunit RPS-12 (Fig 1C; Fig S2C), a significant fraction of ceRACK-1 remains associated to the *let-7* complex (Fig 1C). Therefore, our findings provide evidence that free miRISC interacts with the 40S ribosomal subunit and ceRACK-1 contributes to this interaction.

rack-1 is important for miRNA function in C. elegans

In *C. elegans*, the miRNA pathway plays an important role in the precise control of animal development. The loss-of-function of genes associated with this pathway results in pleiotropic phenotypes, probably reflecting their roles in the activity of all *C. elegans* miRNAs (Bussing et al, 2010; Denli et al, 2004; Grishok et al, 2001; Hammell et al, 2009). To examine whether *rack-1* is important for the miRNA pathway in *C. elegans*, we depleted *rack-1* in animals using RNAi feeding delivery (animals carrying loss-of-function alleles of *rack-1* gene are embryonic lethal; data not shown). While the depletion of the 40S ribosomal subunit *rps-12* leads mostly to embryonic and larval lethality (data not shown), *rack-1(RNAi)* displays developmental timing delay including heterochronic phenotypes that include defects in adult alae (Fig 1D, E and F). We also observed a significant proportion of *rack-1(RNAi)* animals burst from the vulval opening after L4 molt, a phenotype characteristic of *let-7* family miRNA mutants (Abbott et al, 2005; Fig 1D). All these phenotypes are very similar to what we observed in *alg-1(RNAi)* animals and are enhanced in *alg-2(lf)* single mutant animals (Fig 1D). Therefore, the similarity of

the phenotypes observed by the loss of function of *rack-1* and by the depletion of core components of the miRNA pathway, the synergy observed with *alg-2*, the other Argonaute essential for this pathway in *C. elegans* (Grishok et al, 2001), strongly supports the conclusion that *rack-1* functions in the *C. elegans* miRNA pathway.

To better understand the function of *rack-1* in the miRNA pathway, we decided to monitor the miRNA levels of *C. elegans* animals subjected to *rack-1* RNAi. While as recently reported (Kato et al, 2009), we observed a significant decrease in the amount of miRNAs in *alg-1(RNAi)* animals (Fig 2A), the knockdown of *rack-1* in *C. elegans* led to an increase of *let-7* and *lin-4* miRNA levels (Fig 2A). Interestingly, this accumulation of miRNAs was significantly attenuated in the absence of *alg-1* but was unaffected in *alg-2(lf)* animals (Fig 2B). This observation indicates that the accumulation of miRNAs observed in *rack-1(RNAi)* animals requires the Argonaute ALG-1 but not ALG-2 to occur.

Although *rack-1* and *alg-1* knockdowns result in similar heterochronic phenotypes (Fig 1D), the opposite effect observed on miRNA levels suggests that ALG-1 and ceRACK-1 are not acting at the same point in the miRNA pathway. It is very likely that RACK1 is not required for loading and stabilization of miRNAs. The simplest explanation of the accumulation of miRNAs in *rack-1(RNAi)* animals is that the lack of RACK1, which causes failed regulation, could result in a slower turnover of assembled miRISCs. Interestingly, it has been recently reported that the interaction between the miRNA and its mRNA target leads to the degradation of the miRNA (Ameres et al, 2010). Thus, the

accumulation of miRNAs observed in absence of ceRACK-1 might reflect that a significant amount of ALG-1-miRNA complex cannot reach its mRNA target and thus lead to miRNA accumulation.

RACK1 interacts with the human miRISC

Because RACK1 is a highly conserved protein in eukaryotes (Fig S3), we next asked whether RACK1 associates with Argonautes in mammals. While we detected ribosomal RNA, RPS3, a 40S ribosomal protein, human Ago2 and miRNAs such as *let-7* and *miR-21* in the RACK1 immunoprecipitate from HeLa cell lysate, we did not observe an interaction between Ago1 and RACK1 (Fig 3; [Fig S2D](#); [Fig S2E](#)). This observation is surprising since Ago1 is a member of the Argonaute gene family able to bind miRNAs (Liu et al, 2004) and also involved in translational repression. However, the exact mechanism of its action, which could be different from Ago2, is not yet known (Schmitter et al, 2006). The treatment of the samples with RNase A does not completely abrogate the association between RACK1 and the miRISC suggesting that part of the interaction is either direct or mediated by other proteins (Fig 3B; Fig S2D). Thus, as observed in *C. elegans*, components of the mammalian miRISC interact *in vivo* with RACK1.

Human miRNA gene silencing requires RACK1

Next, we tested whether RACK1 is required for miRNA-mediated translational repression in mammalian cells. When cells were treated with siRNA targeting RACK1, we observed a marked increase in the expression of the endogenous IMP-1 and RAS

proteins: two characterized *let-7* targets in human cells (Johnson et al, 2005; Selbach et al, 2008; Fig 4A). However, RACK1 knockdown did not alter the steady state level of IMP-1 mRNA, suggesting post-transcriptional regulation (Fig S4A). To demonstrate that the effect of RACK1 on translation requires miRNAs, we carried out dual luciferase assays with a miRNA-regulated reporter construct that contains eight tandem *let-7* sites (Iwasaki et al, 2009) as well as a luciferase reporter that contains part of the *let-7* targeted HMGA2 3'UTR. The knockdown of RACK1 with three independent siRNAs significantly and specifically altered the expression of both luciferase reporters (Fig 4B and Fig S5). To further show that this affect is specific to RACK1, we altered the expression of the human 40S ribosomal subunit RPS3. The knockdown of RPS3 resulted in a general inhibition of translation without any specific affect on the miRNA targeted luciferase reporter (Fig S6). Also, when we used a reporter that contains three perfect complementary target sites to endogenous *let-7a*, we observed no significant change in the luciferase expression (Fig S7). Therefore, RACK1 is required to mediate miRNA-dependent translational repression but it is not required for RNAi.

Alteration of RACK1 levels impairs miRNA and Argonaute association with translating ribosomes

Because RACK1 has been previously identified as a core component of the ribosome (reviewed in Nilsson et al, 2004), we then decided to test whether RACK1 contributes to the recruitment of miRISC to the translational machinery. We carried out sucrose gradient fractionation to monitor distribution of the *let-7* miRNA in *rack-1(RNAi)* and

control(RNAi) animals. While the polysome distribution is not significantly affected in the *rack-1(RNAi)* population (Fig S8C) nor is the distribution of *let-7* mRNA targets such as *lin-41* and *daf-12* (Reinhart et al, 2000; Slack et al, 2000; Vella et al, 2004; Grosshans et al, 2005; Fig 5A), the amount of *let-7* miRNA associated to polysomes is significantly reduced when compared to *control(RNAi)* animals (Fig 5A; $p < 0.005$).

Human Argonautes have been shown to be associated with polysomes, as their association with heavy fractions is abrogated by puromycin treatment (Nottrott et al, 2006). We have also shown that hAgo2 co-fractionate with RACK1 on the polysomes using sucrose gradient fractionation approach (Fig S2F). We ~~therefore~~ further tested whether the presence of human Ago2 with polysomes requires RACK1. To monitor the amount of Ago2 associated to ribosomes, we pelleted ribosomal complexes via a sucrose cushion (Halbeisen et al, 2009). When compared to the control, the amount of Ago2 co-pelleted with ribosomes is significantly decreased when RACK1 is depleted by RNAi (Fig 5B; Fig S8B), suggesting that RACK1 contributes to the recruitment of Ago2 to ribosomes.

These two sets of data highly suggest that RACK1 contributes to miRNA-mediated gene regulation at a post-initiation step. Indeed, RACK1 is a stoichiometric component of the 40S ribosome that is perfectly positioned at the exit channel to mediate these types of regulations (Coyle et al, 2009; Sengupta et al, 2004). This could explain the mechanism how miRNA regulate target gene expression at the elongation step (Kim et al, 2004; Maroney et al, 2006; Nelson et al, 2004; Nottrott et al, 2006; Olsen & Ambros, 1999) and

how miRNAs could regulate through target sites in coding regions (Hafner et al, 2010). In *S. cerevisiae*, RACK-1 is required to recruit Scp160p to specific mRNAs and thus modulate their translations (Baum et al, 2004). These observations along with our results suggest that RACK1 acts as an evolutionarily conserved molecular adaptor on ribosomes to recruit a variety of regulators of mRNA translation, such as miRISC, and facilitates their interactions with the translational machinery at diverse steps of translation. The RACK1 dependent miRNA-mediated gene regulation is very likely differ from the canonical GW182 dependent miRNA-mediated gene regulation and it may also offer a false safe mechanism that could capture miRNA targeted mRNAs that ~~avoided~~escape regulation at the initiation step.

Methods

Nematode methods

C. elegans strains were grown under standard conditions (Brenner, 1974). Transgenic lines MJS10 and MJS17 were produced by microinjection as described in (Mello & Fire, 1995). RNAi experiments were performed with synchronized animal population as previously described in (Chendrimada et al, 2007).

***C. elegans* polysomes fractionation**

C. elegans polysomes fractionation was performed as originally described by Ding and Grosshans (Ding & Grosshans, 2009). Quantitative detection of mRNAs and miRNAs

were performed using TaqMan Gene Expression and microRNA Assay kits (Applied Biosystems), respectively.

Human cells sucrose cushion

HeLa lysates (lysis buffer: 1% NP40, 10 mM HEPES [pH 7.4], 150 mM KCl, 5 mM MgCl₂, 0.25 mM DTT, 50 μM cycloheximide, 0.4 U/ul RNAsin and protease inhibitors) were pretreated with cyclohexamide, spun down (10,000 X g, 10 minutes, 4°C), and equivalent amounts of supernatant were layered onto a 0.5 M sucrose cushion. Samples were then spun at 107,400 X g for 45 minutes at 4°C in an Optima Max Ultracentrifuge.

siRNA transfection in Human cells

Cells were plated at 1.25×10^5 per well of a 6-well plate. For each well 200 pmol of siRNA was diluted in 175 μl of Opti-MEM (GIBCO). 2 μl of oligofectamine (Invitrogen) was diluted in 13 μl Opti-MEM and incubated at room temperature for 5 minutes. The siRNA mixture and the oligofectamine mixture were mixed and incubated at room temperature for 20 minutes. The sample was then added to the well and mixed gently. The media was replaced after 4-6 hours.

Acknowledgments

We would like to thank Dr Victor Ambros, Dr Rhea Utley, Andrew Sobala and members of our laboratories for comments on the manuscript. We also like to thank Dr Gunter Meister for hAgo1 and hAgo2 antibodies and Eric Paquet for statistical support. Some nematode strains were provided by the *Caenorhabditis* Genetics Center, which is funded

by the NIH National Center for Research Resources (NCRR). The work has been funded by the Wellcome Trust and European Framework 6 SIROCCO consortium fund (G.H.) and the Canadian Institutes of Health Research (CIHR) (M.J.S.). S.B. is a participant of the Wellcome Trust PhD program. G.H. is a Wellcome Trust CD fellow and M.J.S. is a CIHR New Investigator.

Supplementary information is available at *EMBO reports* online.

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Figure legends

Figure 1: RACK1 interacts with constituents of *Caenorhabditis elegans* and human miRISC. (A) **Recombinant ceRACK-1 interacts with GST tagged ALG-1.** Western blot analysis of GST pulldowns of His-tagged ceRACK-1 incubated with GST or GST-ALG-1 and probed with anti-His antibody. Input represents the equivalent of 10% of the His-RACK-1 used for pulldown. (B) **ceRACK-1 associates with the miRISC in *C. elegans*.** Extracts from MJS10 strain were incubated with the *let-7*-complementary (*let-7*), *lin-4*-complementary (*lin-4*), or non-specific (*ctl*) tethered 2'-*O*-methyl oligonucleotide. Beads were washed, and bound GFP::*ALG-1* and HA::ceRACK-1 were detected by western blotting. (C) **Interaction of ceRACK-1 but not RPS-12 with the miRISC is partially resistant to RNase treatment.** Strains expressing tagged proteins were incubated with the *let-7*-complementary (*let-7*) or non-specific (control) tethered 2'-*O*-methyl oligonucleotide. Beads were washed and treated (+) or not (-) with RNase followed by the detection of GFP::*ALG-1*, HA::ceRACK-1 and HA::RPS-12 by western blotting. In B and C, input represents the equivalent of 5% of the total extract incubated with tethered oligonucleotides. Dashed lines indicate that unrelated lanes have been removed between samples. Relative intensities of the signal are shown under each lane in panels C and D. (D) **Knockdown of ceRACK-1 causes heterochronic defects similar**

to those observed for *alg-1(RNAi)*. Adult alae defects and bursting vulva were scored on young adult animals fed bacteria expressing either control, *rack-1* or *alg-1* dsRNA. Developmental (dev.) defects observed include one or more of the following: larval lethality, molting defects, vulva and gonadal morphogenesis defects. Number (n) of animals scored is indicated. Asterisk (*): Since *alg-2(lf); rack-1(RNAi)* animals exhibit severe larval lethality and developmental arrest, sufficient numbers of adult stage animals could not be obtained to reliably scored vulval bursting (NA), however alae defects were scored for sixty animals that managed to reach the adult stage. ND: not determined. **(E)** Representative adult alae defect (Nomarski optics) observed in *rack-1(RNAi)* animals. The white bars show region of adult cuticules lacking alae, or with abnormal alae morphology. Magnification is 630x. **(F)** Extra seam cells (top panel) and defect in seam cell fusions (arrows; bottom panel) are observed in adult *rack-1(RNAi)* animals. The magnification of both pictures is 1,000x.

Figure 2: ceRACK-1 affects level of miRNAs in an *alg-1* dependent manner. (A) While *alg-1(RNAi)* reduces miRNA levels, *rack-1(RNAi)* leads to an accumulation of miRNAs. (B) ALG-1 but not ALG-2 is important to accumulate miRNAs in *rack-1(RNAi)* animals. The miRNA levels were measured relative to the small nucleolar RNA (*sn2841*) using quantitative RT-PCR (TaqMan Assays) in adult animals fed with bacteria expressing either control (*ctl*), *alg-1* or *rack-1* dsRNA, as indicated. In all RNAi conditions, no significant changes are observed in the level of the control RNA *sn2841* (data not shown). The error bars represent the 95% confidence interval from three

independent experiments and a Student's two-sided *t*-test was applied on the normalized c_t values to obtain *p*-values.

Figure 3: RACK1 interacts with hAgo2 and miRNAs.

(A) Human RACK1 binds to both Ago2 and miRNAs. RACK1 was immunoprecipitated with monoclonal RACK1 antibody (RACK1) and non-conjugated Protein A beads as a negative control. Input represents the equivalent of 4% of the total extract used for immunoprecipitation. The immunoprecipitate was tested for the presence of RACK1, hAgo2 and hAgo1 by western blotting and *let-7* miRNA by Northern hybridization. **(B) RACK1 interaction with human Ago2 and the miRNA *miR-21* is RNA independent.** Immunoprecipitations were performed with mouse monoclonal antibodies raised against RACK1 and GFP (used as a negative control) as indicated. Input represents 10% of total lysate used for the immunoprecipitation. The immunoprecipitates were treated with RNase A for 1 hour at 4°C. The supernatant (S) and the immunoprecipitate (B) samples, +/- RNase treatment, were tested for the presence of Ago2, RACK1 and *miR-21*.

Figure 4: RACK1 is required for miRNA silencing in human cells. (A) RACK1 is required for the silencing of the human IMP-1 and RAS. Lysates from RNAi treated cells were immunoblotted with RACK1, IMP-1 and RAS antibodies, as indicated. Tubulin immunoblot served as a loading control. **(B) RACK1 affects the translation of a reporter that carries miRNA target sites.** Renilla luciferase constructs that contain a 3' UTR with either eight *let-7* miRNA target sites or no *let-7* miRNA target sites were

transfected in RNAi treated HeLa cells. Firefly luciferase was used as an internal control. The graph represents the quantification of the dual luciferase assay from five repeats and the error bars represent standard propagated errors and significance were analyzed with a Student's *t*-test. RL:8 *let-7* sites: reporter contains eight tandem *let-7* miRNA target sites. RL:no *let-7* site: reporter without *let-7* miRNA site. Upper panel, detection of RACK1 shows the efficiency of the knockdown. Tubulin was used as a loading control.

Figure 5: RACK1 is important to recruit the miRISC to translating ribosomes. (A) The level of *let-7* and *let-7* targets, *lin-41* and *daf-12*, in each fractionation collected from the sucrose gradient was monitored by quantitative RT-PCR. Mean fold change of *let-7* and mRNA target RNA molecules associated to polysomes fractions of *rack-1* (RNAi) animals relative to animals exposed to control (*ctl*) is shown. Two independent experiments were performed in replicates. Student's two-sided *t*-test was used to assess the significance of the polysomal distribution of *let-7* miRNA between *control(RNAi)* and *rack-1(RNAi)* animals. Prior to the *t*-test, the Shapiro-Wilk test confirmed that the data were normally distributed. (B) Ribosomal proteins were pelleted with a sucrose cushion from HeLa cells that were treated with control or RACK1 siRNAs and the pellet associated Ago2 was quantified and presented as a ratio of the sum of the free and polysome associated Ago2. The graph is derived from five independent experiments carried out with three independent RACK1 siRNAs. Error bars represent the standard deviation and a Student's *t*-test was performed to determine the significance of the data.