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***Drosophila* Argonaute2 turnover is regulated by the ubiquitin proteasome pathway**

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Abstract

Argonaute (AGO) proteins play a central role in the RNA interference (RNAi) pathway, which is a cytoplasmic mechanism important for post-transcriptional regulation of gene expression. In *Drosophila*, AGO2 also functions in the nucleus to regulate chromatin insulator activity and transcription. Although there are a number of studies focused on AGO2 function, the regulation of AGO2 turnover is not well understood. We found that mutation of T1149 or R1158 in the conserved PIWI domain causes AGO2 protein instability, but only T1149 affects RNAi activity. Mass spec analysis shows that several proteasome components co-purify with both wildtype and mutant AGO2, and knockdown of two proteasome pathway components results in AGO2 protein accumulation. Finally, AGO2 protein levels increase after treatment with the proteasome inhibitor MG132. Our results indicate that the ubiquitin-proteasome pathway is involved in AGO2 protein turnover.

Keywords

Argonaute; RNAi; protein stability; ubiquitin; proteasome

Introduction

The functions of evolutionarily conserved AGO family proteins have been reported in different species. AGO proteins are best known for their effector role in RNA silencing to control gene expression. AGO protein binds small RNA, and this complex associates with a homologous target mRNA to induce degradation or inhibition of translation [1]. Due to its central role in RNA silencing, AGO proteins mainly localize in the cytoplasm. However, AGO proteins also function in the nucleus. In fission yeast, RNAi nucleates centromeric heterochromatin formation [2]. In *Drosophila*, AGO2 interacts with chromatin insulator proteins and regulates chromatin looping in an RNAi-independent manner [3]. Both *Drosophila* AGO2 and its mammalian ortholog AGO1 also regulate alternative splicing [4]

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[5] [6] [7]. It is expected that Argonautes play various roles in the cell in different organisms.

Previous work has identified several interacting proteins as well as posttranslational modifications of AGO proteins that affect their stability. P700 hydroxylation of human AGO2 is important for association of HSP90, and mutation of P700 causes AGO2 instability [8] [9]. Furthermore, SUMOylation also affects human AGO2 protein stability [10] [11]. In *Drosophila*, Aubergine and AGO3, which belong to the PIWI subclass of Argonautes, are methylated on arginine residues by protein methyltransferase 5 (PRMT5), and this modification is important for protein stability [12]. Interestingly, Smibert et al. found that stability of the *Drosophila* microRNA pathway-specific AGO1 protein is regulated by the activity of the miRNA pathway itself [13]. Knockdown of key miRNA pathway genes *Dcr1*, *Pasha*, or *droscha* reduces AGO1 protein levels, but this effect can be rescued by supplementation of Dicer1-independent transgenic miRNAs. These results indicate that AGO1 stability is regulated by the presence of an active miRNA pathway as well as available miRNA levels. Importantly, AGO1 protein degradation is dependent on the ubiquitin proteasome pathway. In contrast, no effect is observed for AGO2 stability in the *Dcr-2* null mutant background, indicating that AGO2 levels are not regulated by the activity of RNAi pathway [13]. However, it is still unclear how AGO2 turnover is regulated.

In this study, we mutated conserved residues in AGO2 and examined effects on protein function, localization, and stability. Mutant forms of AGO2 showed the same localization pattern as wildtype AGO2 but displayed reduced protein instability. In order to identify the responsible factor or pathway, we performed immunoprecipitation of AGO2 followed by LC-MS/MS. We found that many ubiquitin-proteasome related proteins are co-purified with AGO2, and knockdowns of two of these factors led to AGO2 protein accumulation. Finally, treatment with proteasome inhibitor MG132 resulted in both WT and mutant AGO2 protein accumulation. Taken together, our results suggest that AGO2 is degraded by the ubiquitin-proteasome pathway, particularly when destabilized.

Materials and Methods

Transfection of cultured cells

Transfections of 1×10^7 S2 cells using 2 μ g of plasmid/dsRNA were performed using Cell Line Nucleofector Kit V (Amaxa Biosystems) transfection reagent using the G-30 protocol. Cells were grown at 25°C in Shields and Sang M3 insect medium (Sigma) supplemented with 0.1% yeast extract, 0.25% bactopectone and 10% heat inactivated fetal bovine serum. Knockdown efficiencies were confirmed for each experiment by RT-qPCR.

Transgenic fly construction

10.15kb of AGO2 genomic fragment containing promoter/3'UTR region was digested using SacI from RP98-21A13 from BACPAC Resources Center. To construct mutant genomic fragments, a *SmaI/EcoRI* digested 2.8kb fragment was ligated to pBluescript vector and used for site-directed mutagenesis. The mutated fragment was ligated to genomic construct using *SmaI/MluI*. For constructing backbone plasmid, *vermillion* and *attB1* were digested by

HindIII/SpeI from pVALIUM10 plasmid, and this fragment was ligated to pBluescript vector. AGO2 genomic fragment was then ligated to backbone plasmid using blunt-ended *SacI* site by Klenow treatment. Plasmids were injected to *yv; attP40* by Genetic Services, Inc. Transgene positive flies were selected by *vermilion*⁺ eye color.

Immunostaining

Harvested cells were washed with PBS and re-suspended in PBS. Cells were applied to 0.01% poly-L-lysine coated multi-well slides. After aspiration of excess cells, cells were fixed with 4% paraformaldehyde in PBS with 0.1% Triton X-100 for 30 min. Cells were blocked with PBS plus 0.1% Triton X-100 and 5% BSA for 30 min. Primary antibody with blocking solution (PBS plus 0.1% Triton X-100, 10% Normal Goat Serum) was incubated with cells overnight at 4°C. Slides were washed three times with PBS plus 0.1% TritonX-100 for 15 min. Secondary antibody in blocking solution was incubated with cells for 2 h at 37°C. Slides were washed three times with PBS plus 0.1% TritonX-100 for 15 min and stained with DAPI (0.1µg/mL in PBS). Antibody 4D2 was used as is with 0.1% TritonX-100, 9D6 as is with 0.1% TritonX-100, anti-FLAG M2 (1:1000), and secondary antibody Alexa Fluor 488 goat anti-mouse IgG was used at 1:1000.

Cycloheximide/MG132 treatment

Cycloheximide (Sigma) or MG132 (Sigma) were dissolved in cell culture medium and added to cells at a final concentration of 100 µg /mL and 10µg /µL of each, respectively.

Immunoprecipitation

S2 cells were harvested 4 d after transfection and lysed using RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% DOC, 0.1% SDS, containing protease inhibitor cocktail, Roche). EZview red anti-FLAG M2 conjugated affinity gel (Sigma) was washed two times with PBS, one time with RIPA buffer, and then added to lysate. Lysates with added antibody were rotated at 4°C overnight, and immunoprecipitates were washed three times with RIPA buffer. Immunoprecipitation was confirmed by Western blotting.

Antibodies

For Western blotting, polyclonal rabbit Anti-Ubiquitin (DAKO, z0458, 1:250), monoclonal Anti-FLAG (Sigma, M2, 1:1000), rabbit anti-AGO2 (gift from Q. Liu [14], 1:2,000), and mouse monoclonal anti-Tubulin antibody (Sigma, 1:500,000) were used. For immunostaining, mouse anti-AGO2 (4D2, 1:1,000 and 9D6, as is, gift from M. Siomi and H. Siomi) were used.

Mass spectrometry

Copurified proteins were precipitated using trichloroacetic acid, washed with acetone, and dried. Digestion of copurified proteins with trypsin and LC-MS/MS analysis was performed by Taplin Mass Spectrometry Facility at Harvard Medical School.

RT-qPCR

Total RNAs were isolated using TRIzol reagent by the manufacturer's recommended protocol. 1–2 µg of total RNA was used for reverse transcription using Oligo (dT)₂₀ primer and SuperScript III reverse transcriptase (Invitrogen) by the recommended protocol. Real-time PCR was performed using HotStart SYBR green qPCR Master Mix (Affymetrix). Transcript levels were quantified in the linear amplification range by calibration to a standard curve of DNA to account for differences in primer amplification efficiencies. Primer sequences are listed in Supplementary Table 1.

In vitro transcription

Synthesis of dsRNA was performed as described previously [3], using PCR amplicons derived from cDNA.

Results

Differential AGO2 cellular distribution revealed by two different specific antibodies

We observed that two specific monoclonal antibodies against *Drosophila* AGO2 produced different cellular localization patterns. Monoclonal 9D6 antibody was developed against the glutamine rich N-terminal domain, and monoclonal 4D2 recognizes 16 amino acid residues (1145–1160) within the conserved PIWI domain (Fig. 1A [15]). Immunostaining using 4D2 antibody shows predominantly cytoplasmic AGO2 localization, consistent with its known RNAi function within this cellular compartment. On the other hand, 9D6 antibody reveals a high proportion of AGO2 within the nucleus (Fig. 1B), and this nuclear signal is specific for AGO2 [3]. Since 9D6 antibody is capable of immunoprecipitating AGO2 bound to small RNA [15], we presume that this antibody is also capable of detecting cytoplasmic AGO2. To reconcile these observations, we hypothesized that the 4D2 epitope region of nuclear AGO2 may be masked by other factor(s). We compared AGO2 amino acid sequences including the 4D2 epitope region from a variety of species using the PROMALS3D multiple sequence and structure alignment server (Fig. 1C). We chose to focus on T1149, Y1150 and R1158 for further study (Fig. 1D).

In vivo effects of AGO2 mutation

In order to study the requirement for particular residues within the 4D2 recognition region, we constructed point mutations in *AGO2* and generated mutant transgenic flies. We cloned genomic versions of WT, *AGO2^{m1}* (T1149A, Y1150A) and *AGO2^{m2}* (R1158A) mutant transgenes under the control of the endogenous promoter and inserted them into the *attP40* landing site using the PhiC31 system [16]. We first tested mutant *AGO2* RNAi function using a *GMR-wIR* reporter, which expresses a 629bp perfect hairpin dsRNA homologous to the endogenous *white* gene, resulting in its silencing [17]. In the *AGO2^{51B}* null mutant, RNAi function is lost; therefore, flies exhibit red eye color (Fig. 2A, top left panel). Expression of one copy of the WT *AGO2* transgene in the *AGO2^{51B}* null genetic background rescues silencing, which results in very light, variegated eye pigmentation (Fig. 2A left, bottom). However, the eyes of *AGO2^{m1}* mutants are light red, indicating that *AGO2^{m1}* can only partially rescue RNAi function of the null mutant (Fig. 2A, middle). In

contrast, *AGO2^{m2}* flies exhibit the same phenotype as WT transgene, suggesting that the RNAi system works normally in this mutant (Fig. 2A, right).

Western blotting of homozygous WT *AGO2* transgenic flies in the *AGO2^{51B}* null background shows the same expression level as endogenous wildtype AGO2 (Fig. 2B). However, both homozygous *AGO2^{m1}* and *AGO2^{m2}* display equal but lower expression than WT. We conclude that this lower than endogenous level of expression is adequate for RNAi but that the *AGO2^{m1}* mutant is additionally defective for RNAi activity.

Subcellular localization of AGO2 point mutants

One possibility for *AGO2^{m1}* mutants being reduced for RNAi activity is due potentially to altered subcellular localization of AGO2. We generated additional isolated single mutants *AGO2^{m3}* (T1149A) and *AGO2^{m4}* (Y1150A) and fused each construct with the FLAG/HA epitope at the N-terminus. We transfected S2 cells expressing each construct under the *Act5c* promoter and performed immunostaining using anti-FLAG antibody. As shown in Fig. 2C, WT AGO2 is predominantly cytoplasmic, and no difference in localization was observed among WT and *AGO2^{m1-m4}* mutants. Therefore, these results suggest that T1149A, Y1150A and R1158A mutations do not affect AGO2 steady-state cellular distribution.

T1149A mutation affects AGO2 protein stability

Both mutant transgenic *AGO2^{m1}* and *AGO2^{m2}* proteins showed lower expression than WT *in vivo*; therefore, we hypothesized that these mutations could affect protein stability. In order to investigate this possibility, we performed cycloheximide (CHX) chase assays to inhibit translation and monitor protein turnover. Plasmid transfected S2 cells were treated with CHX, and cells were harvested at indicated times (Fig. 3A). WT AGO2 protein was slowly degraded but still detectable by α -FLAG Western blotting after 24 h. On the other hand, *AGO2^{m1}* protein could not be detected after 12 h, indicating that *AGO2^{m1}* turnover is faster than WT. The half life of *AGO2^{m3}* is the same as that of *AGO2^{m1}*, but *AGO2^{m4}* turned over at the same rate as WT. These results suggest that T1149A mutation causes AGO2 instability. *AGO2^{m2}* (R1158A) also shows reduced AGO2 stability but to a lesser extent than *AGO2^{m1}*.

Identification of AGO2 interacting proteins that potentially regulate protein stability

We hypothesized two mechanisms for accelerated mutant *AGO2^{m1}* turnover. First, an unknown protein may ectopically interact with mutant *AGO2^{m1}* and recruit it to degradation. Second, an unknown protein may bind the wildtype AGO2 PIWI domain near T1149 and/or Y1150 residues and protect AGO2 from degradation. In the second scenario, the *AGO2^{m1}* mutant would be reduced in interaction with this putative protective factor. In order to identify AGO2-interacting proteins that potentially affect AGO2 stability, we performed immunoprecipitation of transfected tagged WT and *AGO2^{m1}* mutant followed by mass spec analysis. FLAG/HA fused AGO2 was expressed using the *Act5c* promoter in S2 cells and immunoprecipitated from total cell extract using anti-FLAG antibody (Fig. 3B). As a negative control, we also transfected empty vector and performed the identical immunoprecipitation in parallel. More WT than *AGO2^{m1}* was purified in AGO2 transfected cells, while no AGO2 was purified in the negative control. Mass spec analysis confirmed

that RNAi and nonsense mediated decay (NMD) related proteins are co-purified with both wildtype and mutant AGO2 (Supplemental Table 2).

We found that many components of the ubiquitin-proteasome pathway were co-purified with both WT and AGO2^{m1}. These include the 19S (Rpn9, Rpn11, Rpn8, etc.) and 20S (α 7, α 1, α 6, α 4, etc.) proteasome subunits. The 20S proteasome component α 7 (Prosa7) was more abundant in the AGO2^{m1} purification but was also isolated in conjunction with WT AGO2. Furthermore, Uev1A, an E2 ubiquitin-conjugating enzyme [18], was co-purified with AGO2. To test the effect of *Prosa7* or *Uev1A* knockdown on AGO2 protein stability, we transfected dsRNA and plasmid expressing FLAG-AGO2 into S2 cells and examined AGO2 stability. Knockdown efficiency was confirmed by RT-qPCR (Fig. 3C). Moreover, when *Prosa7* was depleted, FLAG-AGO2 levels increase. We detected higher molecular weight bands above FLAG-AGO2 (Fig. 3D, asterisk) in *Uev1A* knockdowns, and these species also accumulated after knockdown of *Prosa7*. Western blotting using anti-ubiquitin also shows higher molecular weight bands in the same vicinity; therefore, AGO2 may be ubiquitinated in response to depletion of these proteasome pathway factors.

Uev1A is involved in AGO2 protein turnover

We also examined whether endogenous AGO2 protein stability is increased by *Uev1A* knockdown. Higher molecular weight species reactive with AGO2 antibodies are observed after Uev1A knockdown (Fig. 4A), but AGO2 transcript abundance is not increased by *Uev1A* knockdown (Fig. 4B). Therefore, the observed effect is likely due either to improvement of AGO2 protein stability or inhibition of AGO2 protein degradation. In order to confirm involvement of the proteasome pathway in AGO2 turnover, we treated cells with CHX and MG132 proteasome inhibitor for 12 h. Levels of both AGO2 WT and mutant proteins increase after treatment with MG132 (Fig. 4C). These results suggest that the ubiquitin-proteasome pathway is involved AGO2 protein turnover.

Discussion

In this study, we investigated the effect of mutations in conserved amino acids within the PIWI domain on AGO2 localization and function. Based on immunostaining results, mutation of T1149, Y1150, and R1158 residues in the 4D2 epitope region do not affect AGO2 cellular distribution. However, we found that T1149 and R1158 mutations cause AGO2 protein instability. We showed that AGO2 protein accumulates after treatment with proteasome inhibitor or knockdown of proteasome pathway components, suggesting that the proteasome pathway is involved in AGO2 turnover.

Previous work in human cells has shown that the HSP90 co-chaperone affects AGO1 and AGO2 stability. HSP90 is essential for small RNA loading into the RISC complex, and inhibition of HSP90 by geldanamycin decreases protein levels of AGO1 and AGO2 [19]. The resultant decreases of AGO1 and AGO2 levels were alleviated by addition of MG132, suggesting that HSP90 protects AGO proteins from degradation by the proteasome pathway [19]. However, interaction of Rat AGO protein GERP95 with HSP90 was shown to be dependent on the GERP95 N-terminus and not the PIWI domain [20]. Since the *Drosophila* AGO2 T1149A and R1158A mutations are located within PIWI domain, we do not

anticipate that interaction with HSP90 is disrupted in these mutants [20]. Nevertheless, HSP90 was observed in our mass spec analyses; therefore, it remains possible that *Drosophila* HSP90 also regulates AGO2 protein stability through the ubiquitin-proteasome pathway.

Our mass spec data revealed that many ubiquitin-proteasome related proteins are co-purified with AGO2. Both WT and m1 mutant forms of AGO2 copurify with proteasome or related factors. Prosa7 was more abundant in AGO2^{m1} than WT purifications, suggesting that AGO2^{m1} may be accelerated for degradation compared to WT, perhaps due to misfolding of the protein. We also found the E2 ubiquitin ligase Uev1A co-purified with both mutant and WT AGO2, and knockdown of *Uev1A* slightly increases higher molecular weight AGO2 protein levels. Since Uev1A is an E2 ubiquitin conjugating enzyme, AGO2 is unlikely to be a direct target of Uev1A. Uev1A functions in the Imd immune response pathway, which activates NF- κ B and regulates activation of immunity [21]. The RNAi machinery is also involved in antiviral immunity. When viral dsRNAs are produced in infected cells, processed siRNAs are loaded into AGO2 for degradation of viral transcripts [22]. However, the details of the RNAi response as an immune system is still not well understood. We speculate that the change in AGO2 protein levels after *Uev1A* knockdown may be a feedback mechanism of immune response signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Mutation of the PIWI domain in *Drosophila* Argonaute2 (AGO2) causes protein instability.
- Ubiquitin proteasome components co-purify with AGO2.
- The turnover of AGO2 is regulated by the ubiquitin proteasome pathway.

Fig. 1. Differential AGO2 localization patterns between 9D6 and 4D2 immunostaining
 (A) *Drosophila* AGO2 protein domains. Bold lines indicate recognition region of AGO2 antibodies. (B) Immunostaining was performed using mouse monoclonal antibody 4D2 or 9D6 and a secondary antibody against mouse IgG conjugated to Alexa Fluor 488 (green, left panel) in S2 cells. DAPI staining (blue, middle panel) and merged signals (right panel) are shown. (C) Amino acid sequences including 4D2 epitope region were compared using PROMALS3D multiple sequence and structure alignment server. Asterisks indicate residues mutated to alanine. Hs; *Homo sapiens*, Dm; *Drosophila melanogaster*. (D) Specific mutations in m1-m4 mutants.

Fig. 2. *In vivo* RNAi activity of transgenic mutant AGO2 flies

(A) *In vivo* RNAi activity test using *GMR-wIR* reporter. Top fly in each panel shows *AGO2*^{51B} null mutant control. RNAi activity of single copy transgene (TG) was tested in *AGO2*^{51B} null mutant background. (B) Homozygous transgene expression in the *AGO2*^{51B} null mutant background. Adult fly heads were used for protein extraction. 4D2 antibody was used for AGO2 Western blotting. Anti-Tubulin was used as a loading control. (C) FLAG-AGO2 plasmids were transfected into S2 cells. After 2 d, cells were collected and immunostaining was performed using mouse anti-FLAG antibody and a secondary antibody against mouse IgG conjugated to Alexa Fluor 488 (green, top panel). DAPI staining (blue, middle panel) and merged signals (bottom panel) are shown.

Fig. 3. Protein instability of mutant AGO2

(A) AGO2 protein stability after treatment with cycloheximide (CHX). Transfected cells were treated with cycloheximide and harvested at the indicated time points for Western blotting of cell lysates. Anti-FLAG was used to monitor transgenic AGO2 and anti-Tubulin was used as a loading control. (B) Silver staining of immunoprecipitated proteins. The arrow head indicates FLAG-AGO2 and asterisks indicate IgG. (C) Knockdown efficiency of targets was confirmed by RT-qPCR. Double stranded RNA against *GFP* was used as a control. Knock down efficiency is shown as a relative value of the control. (D) Transfection of dsRNA and *AGO2^{m1}* plasmid was conducted on S2 cells, and Western blotting was performed on cell lysates.

Fig. 4. Uev1A and proteasome pathway affect AGO2 stability

(A) Endogenous AGO2 protein levels after *Uev1A* knockdown. Arrow points to higher molecular weight species that accumulate in *Uev1A* knockdown cells. *GFP* dsRNA was used as a control. Anti-AGO2 (Q. Liu) was used for Western blotting. (B) AGO2 mRNA levels and knockdown efficiency were confirmed by RT-qPCR. (C) Transfected cells were treated with cycloheximide (CHX) with or without MG132 for 12 h.