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MicroRNA-binding is required for recruitment of human Argonaute 2 to stress granules and P-bodies

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ABSTRACT

Argonaute proteins are the core components of the RNA-induced silencing complex, the central effector of the mammalian RNA interference pathway. In the cytoplasm, they associate with at least two types of cytoplasmic RNA granules; processing bodies and stress granules, which function in mRNA degradation and translational repression, respectively. The significance of Argonaute association with these RNA granules is not entirely clear but it is likely related to their activities within the RNAi pathway. Understanding what regulates targeting of Argonautes to RNA granules may provide clues as to their functions at these organelles. To this end, there are a number of conflicting reports that describe the role of small RNAs in targeting Argonaute proteins in mammalian cells. We employed quantitative microscopic analyses of human Argonaute 2 (hAgo2) mutants to study factors that govern localization of this RNA-binding protein to cytoplasmic RNA granules. We report, for the first time, that hAgo2 is recruited to stress granules as a consequence of its interaction with miRNAs. Moreover, loading of small RNAs onto hAgo2 is not required for its stability, suggesting that a pool of unloaded hAgo2 may exist for extended periods of time in the cytoplasm.

1. Introduction

Argonaute proteins belong to a highly conserved superfamily found in virtually all eukaryotes and are best known for their roles in RNA interference (RNAi). In somatic cells, there are two main types of Argonaute-associated RNAs: short interfering RNAs (siRNAs) and microRNAs (miRNAs) (reviewed in [1]). Nascent siRNAs and miRNAs are transferred to RNA induced silencing complexes (RISC), the core components of which are Argonaute proteins [2]. It is thought that more than 50% of all mammalian genes are controlled post-transcriptionally by miRNA-dependent mechanisms [3].

There are four members of the Argonaute subfamily in humans (AGO1, AGO2, AGO3 and AGO4); all of which are expressed ubiquitously and bind to both miRNAs and siRNAs [1]. Argonaute proteins are identified by three characteristic domains: PAZ, PIWI and MID. The PAZ domain forms an oligonucleotide-binding pocket that preferentially interacts with 2 nt 3′ overhangs of dsRNAs, which are characteristic of the cleavage products of RNase III family members such as Dicer [4–8]. The MID domain serves as a binding pocket for the 5′ phosphate groups of small RNAs [9–13]. The PIWI domain is required for interaction with Dicer [14], adopts an RNase H-like fold and is the catalytic domain of Argonaute proteins [15–17]. The RNase H-like activity is responsible for the endonucleolytic cleavage of mRNAs targeted by base-pairing with perfectly complementary small RNAs [17,18]. Of the four Argonaute isoforms that are expressed in somatic cells, only hAgo2 contains a catalytically active PIWI domain [18,19].

Because of their central roles in controlling gene expression, the activities of Argonaute proteins must be tightly regulated in mammalian cells. Emerging evidence suggests, not surprisingly, that multiple *cis*- and *trans*-acting factors are involved in regulating Argonaute activity. For example, P700 of hAgo2 is modified by hydroxylation, a process that is important for stability of this protein and, ultimately, RNAi activity [20]. Similarly, phosphorylation of S387 is reportedly necessary for targeting hAgo2 to RNA granules; however, it is not known if the phosphorylation status of this residue affects the RNAi activity of hAgo2. Conversely, phosphorylation of Y529 in the MID domain has a well-defined role in regulating Argonaute function. Specifically, phosphorylation of this residue inhibits RNAi by blocking loading of small RNAs onto hAgo2 [21].

Protein activity can also be regulated by localization. To this end, Argonaute proteins are targeted to at least two different RNA granules in the cytoplasm; processing bodies (P-/GW-bodies) and stress granules (reviewed in [22]). P-bodies are present in

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most mammalian cells and contain RNA degrading enzymes. Stress granules form in response to a variety of cellular stresses, contain stalled translation initiation complexes and may act as mRNA triage sites. Recent data suggest that microscopic P-bodies are not required for RNAi but, instead, form as a consequence of RNAi [23]. Conversely, a functional RNAi pathway is required for P-body formation/stability [23–26]. A pool of hAgo2 is targeted to stress granules, but neither the mechanism of targeting nor the functional consequence of this association is well understood [27–29]. However, Detzer et al. speculates that association of hAgo2 with stress granules may lead to its exclusion from the RNAi pathway, resulting in decreased miRNA- and siRNA-mediated post-transcriptional gene silencing [30].

The hypothesis that hAgo2 associates with P-bodies as a consequence of its role in miRNA-mediated gene silencing, is consistent with reports that mutants of hAgo2, which are unable to bind small RNAs, do not localize to P-bodies [21,31]. Furthermore, it was reported that targeting of hAgo2 to stress granules is dependent on the presence of mature miRNAs, based on evidence from Dicer knock-out cell lines [27]. However, the discovery of a Dicer-independent pathway for miRNA biogenesis suggests that miRNAs are present in Dicer knock-out cells [32,33]. In the present study, we demonstrate that mutations that block the ability of hAgo2 to bind miRNAs prevent its recruitment to stress granules as well as P-bodies. Moreover, we report that these mutants are relatively stable, suggesting that unloaded Argonautes can exist in the cytoplasm for extended periods of time.

2. Materials and methods

2.1. Reagents

Dimethyl sulfoxide (DMSO), 50 mM sodium arsenite solution and cycloheximide were from Sigma Aldrich (St Louis, MO). Cycloheximide was prepared at a stock concentration of 10 mg/mL in water. All reagents for mammalian cell culture were obtained from Invitrogen (Carlsbad, CA).

2.2. Antibodies

Human anti-GW was a gift from Dr. M. Fritzler (University of Calgary, Calgary, AB, Canada). Goat polyclonal anti-T-cell-restricted intracellular antigen (TIA) (sc-1751) was from Santa Cruz Biotechnology (Santa Cruz, CA), anti-heterogeneous nuclear ribonucleoprotein U (hnRNP U) (ab10296) was purchased from Abcam (Cambridge, MA) and mouse monoclonal anti-myc (9E10) hybridoma was from ATCC (Manassas, VA). Goat anti-mouse conjugated to Alexa750 (A21037), donkey anti-mouse conjugated to Alex488 (A21202) and chicken anti-goat conjugated to Alexa647 (A21469) were from Invitrogen, donkey anti-human conjugated to Texas Red was from Jackson Immunoresearch Laboratories (West Grove, PA).

2.3. Plasmids

Argonaute mutants (Q633R, H634A, H634P, PAZ9 and PAZ10 in pcDNA3) were obtained from Dr. G.J. Hannon (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). A plasmid encoding a myc-tagged wildtype hAgo2 was constructed by digesting an annealed myc linker (5′-ACT TAA GAT GGA ACA AAA ACT CAT CTC AGA AGA GGA TCT GAA TAT GCA TAC CGG TCA TCA TCA CCA TCA CCA TAT GAA AGC TTC G; 5′CGA AGC TTT CAT ATG GTG ATG GTG ATG ATG ACC GGT ATG CAT ATT CAG ATC CTC TTC TGA GAT GAG TTT TTG TTC CAT CTT AAG T), with Afl II and Hind III and ligating it into pcDNA4/TO-Afl II-GFP-Hind III-hAgo2 (described previously [28]), digested with Afl II and Hind III, in place of the GFP cassette. pcDNA4/TO-myc-hAgo2-Y529F was generated by using pcDNA4/TO-myc-hAgo2 as a template in a PCR reaction with QuikChange II site-directed mutagenesis kit (Agilent, Santa Clara, CA) using the mutagenic primers Y529F-sense (5′-ATC CTG CCC GGC AAG ACG CCC GTG TTC GCC GAG GTC AAA CGC GTG GGA GAC ACG GTG CTG GGG) and Y529F-antisense (5′-CCC CAG CAC CGT GTC TCC CAC GCG TTT GAC CTC GGC GAA CAC GGG CGT CTT GCC GGG CAG GAT). pcDNA4/TO-myc-hAgo2-Y529E was generated by using pcDNA4/TO-myc-hAgo2 as a template in a PCR reaction with QuikChange II site-directed mutagenesis kit (Agilent) using the mutagenic primers Y529E-sense (5′-ATC CTG CCC GGC AAG ACG CCC GTG GAA GCC GAG GTC AAA CGC GTG GGA GAC ACG GTG CTG GGG) and Y529E-antisense (5′-CCC CAG CAC CGT GTC TCC CAC GCG TTT GAC CTC GGC TTC CAC GGG CGT CTT GCC GGG CAG GAT). Sequences of all vectors and mutants was confirmed by both diagnostic restriction endonuclease digestion and sequencing.

2.4. Cell culture and transfection

HeLa cells were cultured in DMEM supplemented with 100 U/mL penicillin/streptomycin, 10% heat-inactivated fetal bovine serum and 10 mM HEPES, pH 7.4, at 37 °C and 5% CO_2. DNA transfections were performed using Lipofectamine 2000 (Invitrogen) and OptiMEM media (Invitrogen). For 12-well plates, a total of 500 ng of plasmid DNA and 0.75 μL transfection reagent were used per well. Where indicated, transfected cells were treated with cycloheximide prior to lysis and immunoblot analyses. Approximately 24 h post-transfection, growth medium was supplemented with cycloheximide to a final concentration of 100 μg/mL. Following addition of cycloheximide, cells were incubated for an additional 12 h at 37 °C.

2.5. Indirect immunofluorescence fluorescence microscopy

Cells on coverslips were washed several times with phosphate-buffered saline (PBS) before fixation for 15 min with 4% paraformaldehyde (Thermo Fisher, Waltham, MA), After fixation, cells were rinsed with PBS, permeabilized with PBS containing 0.2% Triton X-100 (Thermo Fisher) for 2 min, and then rinsed with PBS before blocking with PBS containing 0.1% Tween (Thermo Fisher, PBS-T) and 2% skim milk powder (blocking solution). Samples were incubated for at least 2 h at room temperature with primary antibodies diluted in blocking solution. Following washing in PBS-T, samples were incubated for 1 h at room temperature with secondary antibodies diluted in PBS containing 0.1% Tween-20. After washing in PBS-T, samples were mounted on microscope slides using ProLong Gold with 4,6-diamidino-2-phenylindole (Invitrogen).

Samples were viewed on an Axiovert 200 M (Carl Zeiss, Thornwood, NY) microscope equipped with a spinning disk confocal unit (UltraView ERS) and Volocity software (PerkinElmer Life and Analytical Sciences, Boston, MA). Images were acquired with a C9100-050 EM-charge-coupled device (Hammamatsu Photonics, Hammamatsu City, Japan) digital camera. Images were quantitated using Imaris (Bitplane, St. Paul, MN) and compiled using ImageJ (National Institutes of Health, Bethesda, MD) and Photoshop (Adobe Systems, San Jose, CA) software. In all cases, samples were viewed using a Plan Apochromat 63× objective lens (Carl Zeiss) with a numerical aperature of 1.4. All images were acquired using identical exposure times for each channel as well as identical Z-spacing and an equal number of slices in the Z stack.

2.6. Immunoblotting

Total cell lysates were prepared by adding 5× Laemmli gel loading buffer supplemented with 5% β-mercaptoethanol to cell monolayers that were then scraped from the plates and passed through a 29-gauge needle. Samples were then boiled and resolved in 8% SDS–polyacrylamide gels and transferred to Trans-Blot nitrocellulose transfer medium (Bio-Rad, Hercules, CA). Membranes were stained in a solution of 0.1% Ponceau S (Kodak, Rochester, NY) in 5% acetic acid and subsequently blocked with PBS containing 5% skim milk powder (blocking solution) and incubated in primary antibodies diluted in the blocking solution. After washing, membranes were incubated with secondary antibodies conjugated to Alexa750 and then exposed to an Odyssey infrared imaging system and software (Li-Cor Biosciences, Lincoln, NE). Membranes were scanned at a resolution of 84 μm.

3. Results

3.1. RNA-binding mutants of hAgo2 are not targeted to P-bodies

Indirect immunofluorescence microscopy was used to confirm that our wild type myc-tagged hAgo2 construct is targeted to P-bodies in transfected HeLa cells. Data in Fig. 1A show that myc-hAgo2 associates with discrete cytoplasmic puncta in the cytoplasm of transfected cells (arrowheads). The identity of these puncta as P-bodies was verified by co-staining with an antibody against the well-characterized P-body component GW182 [34]. Previously, the Hannon laboratory reported that mutations in the PAZ domain of hAgo2 that affect binding of small RNAs, disrupts targeting to P-bodies [31]. The PAZ domain is important for recognition of 2 nt 3′ overhangs of small RNAs which result from Dicer processing of double strand RNA substrates. HeLa cells transfected with plasmids encoding two different myc-tagged PAZ mutants of hAgo2 were processed for indirect immunofluorescene microscopy. The results in Fig. 1A and B show that, while GW182-positive P-bodies were present throughout the cytoplasm of both transfected and untransfected cells, the PAZ-9 mutant did not associate with cytoplasmic granules. Similar results were observed with the PAZ10 mutant (data not shown). In contrast, indirect immunofluorescence showed that the slicer-dead mutant of hAgo2, H634P, does localize to GW182-positive P-bodies (Fig. 1A and B). Similar results were observed with two other slicer-dead mutants of hAgo2, H634A and Q633R (data not shown). These results are consistent with a previously published study which suggests that binding to the 3′ ends of small RNAs is required for targeting of hAgo2 to P-bodies [31].

Phosphorylation of Y529 in the MID domain of hAgo2 is thought to negatively regulate binding to the 5′ ends of small RNAs that associate with Argonaute proteins [21]. Mutation of Y529 to an acidic amino acid residue such as glutamic acid interferes with loading of small RNAs binding by sterically hindering interaction of the negatively charged 5′ phosphate of miRNAs with the MID domain of hAgo2. We generated myc-tagged point mutants of Y529 in hAgo2 (Y529E and Y529F) for localization studies. Consistent with previously published results, we observed that changing Y529 to a phosphomimetic glutamic acid residue results in reduced targeting of hAgo2 to P-bodies (Fig. 1A and B). Unexpectedly, the Y529F mutant which has a non-phosphorylatable phenylalanine residue at position 529, did not localize to P-bodies (Fig. 1A and B).

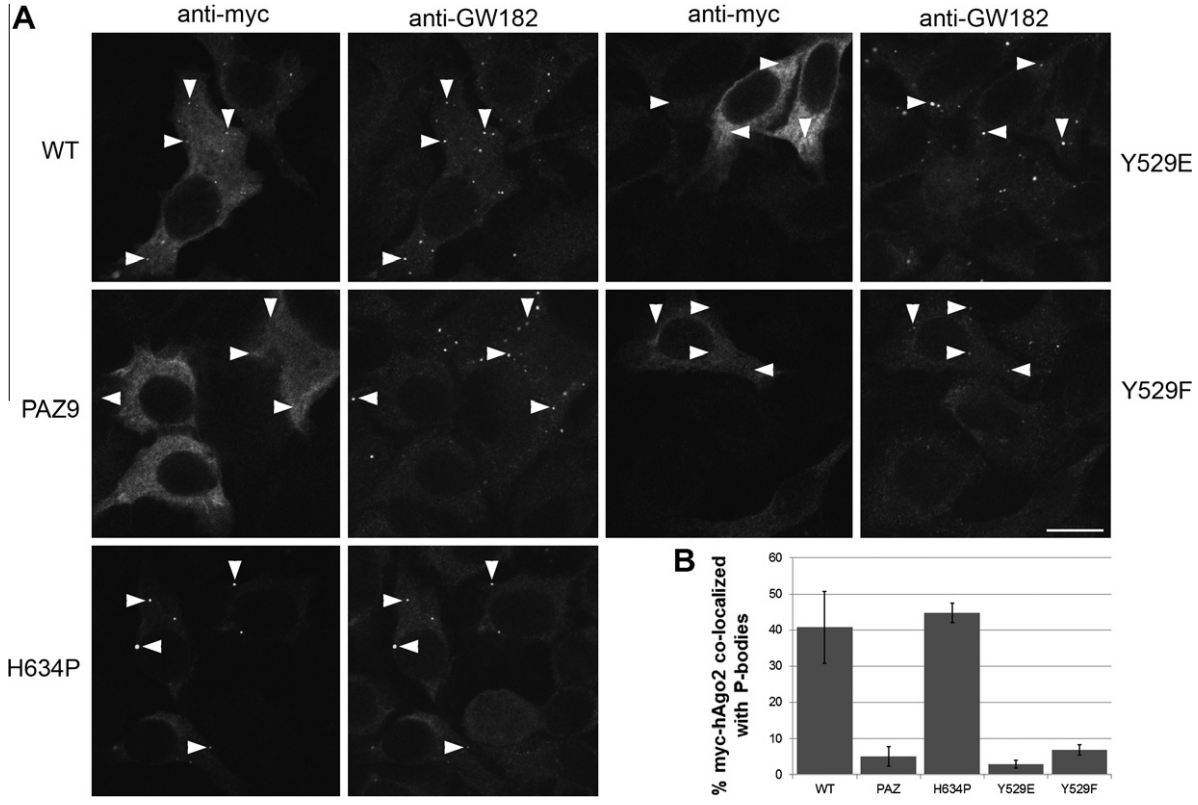

Fig. 1. Mutations in the PAZ and MID domains of hAgo2 that impair small RNA binding, disrupt its localization to P-bodies. Myc-tagged slicer-dead mutants of hAgo2 and wildtype hAgo2 localize to P-bodies. (A) HeLa cells transiently expressing myc-tagged wildtype or mutant hAgo2 proteins were fixed and processed for indirect immunofluorescence. GW182-positive P-bodies (arrowheads) are indicated. Bar = 15 μm. (B) Quantitation of myc-hAgo2 (above a fixed intensity threshold) colocalized with GW182-positive RNA granules. Error bars represent standard error of the mean.

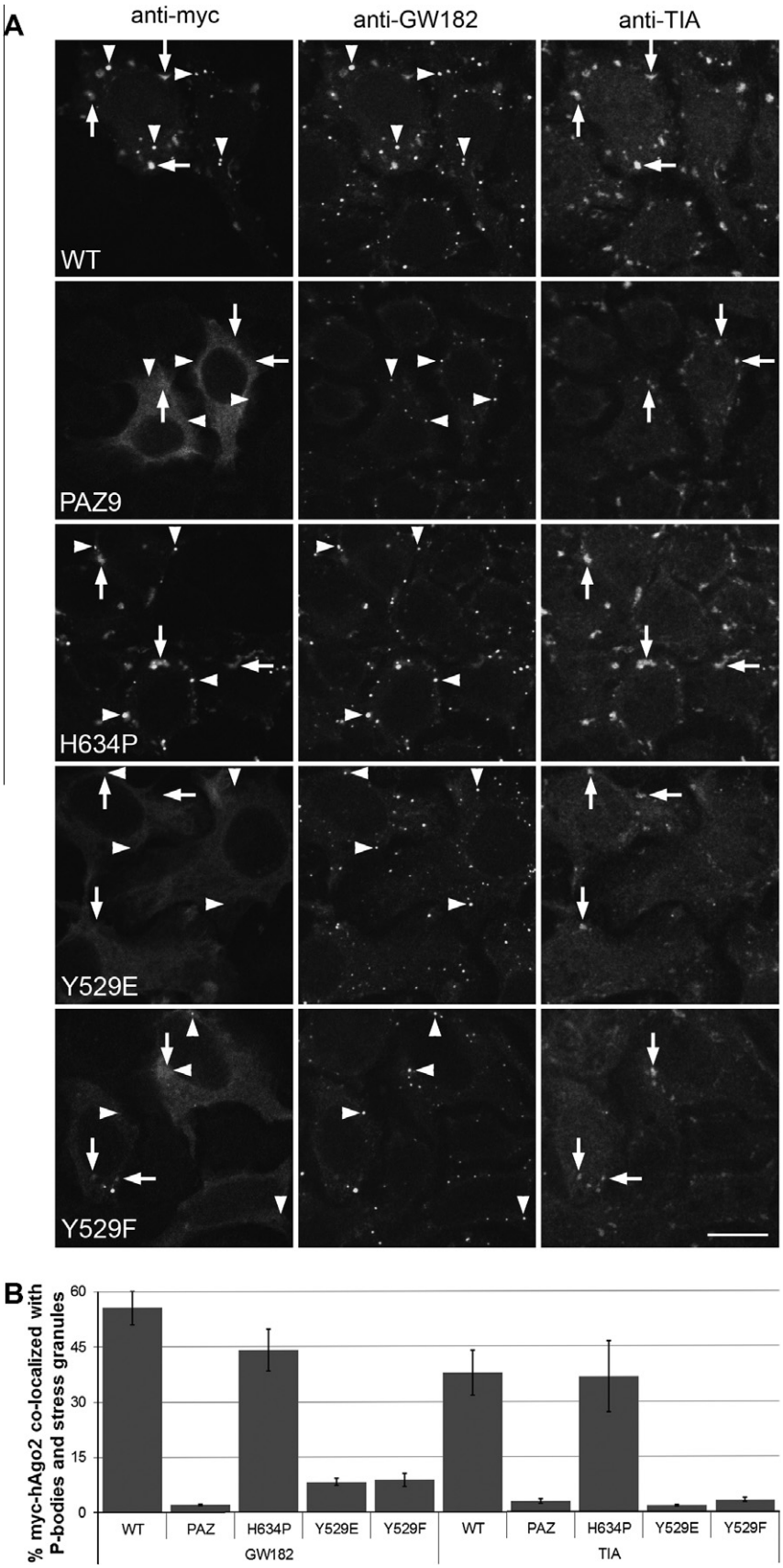

Fig. 2. Mutations in the PAZ and MID domains of hAgo2 that impair small RNA binding, disrupt its localization to stress granules. (A) HeLa cells transiently expressing wildtype or mutant myc-tagged hAgo2 were treated with 500 μM arsenite (+ARS) for 60 min prior to fixation and processing for indirect immunofluorescence. GW182-positive P-bodies (arrowheads) and TIA-1-positive stress granules (arrows) are indicated. It can be seen that similar to myc-tagged wildtype hAgo2, slicer-dead mutants localize to stress granules. Bar = 15 μm. (B) Quantitation of myc-hAgo2 (above a fixed intensity threshold) colocalized with GW182- or TIA-positive RNA granules. Error bars represent standard error of the mean.

3.2. RNA-binding mutants of hAgo2 are not targeted to stress granules

We next investigated how mutations in hAgo2 domains affected association with stress granules. Following treatment with arsenite (+ARS), a large pool of myc-hAgo2 is recruited to TIA-1-positive stress granules in the cytoplasm of HeLa cells (Fig. 2A, arrows). Whereas stress granules formed normally in arsenite-treated cells expressing myc-hAgo2-PAZ-9, no colocalization between this mutant and the stress granule marker TIA-1 was observed (Fig. 2A and B). Similar results were observed with myc-hAgo2-PAZ-10 and when cells were treated with the translational inhibitor hippuristanol (data not shown). Unlike the PAZ mutants, the PIWI domain mutant H634P was efficiently recruited to stress granules (Fig. 2A and B). Similar results were obtained with two other PIWI domain mutants H634A and Q633R (data not shown). Together, these results indicate that the slicer endonuclease activity of hAgo2 is not required for its targeting to stress granules.

Next, the localizations of Y529E and Y529F mutants in arsenite-treated cells were examined. Very little overlap between these hAgo2 mutants and TIA-1-positive stress granules were observed (Fig. 2A and B). Similar results were obtained when cells were treated with hippuristanol (data not shown). These results suggest that dynamic phosphorylation of tyrosine 529 is important for localization of hAgo2.

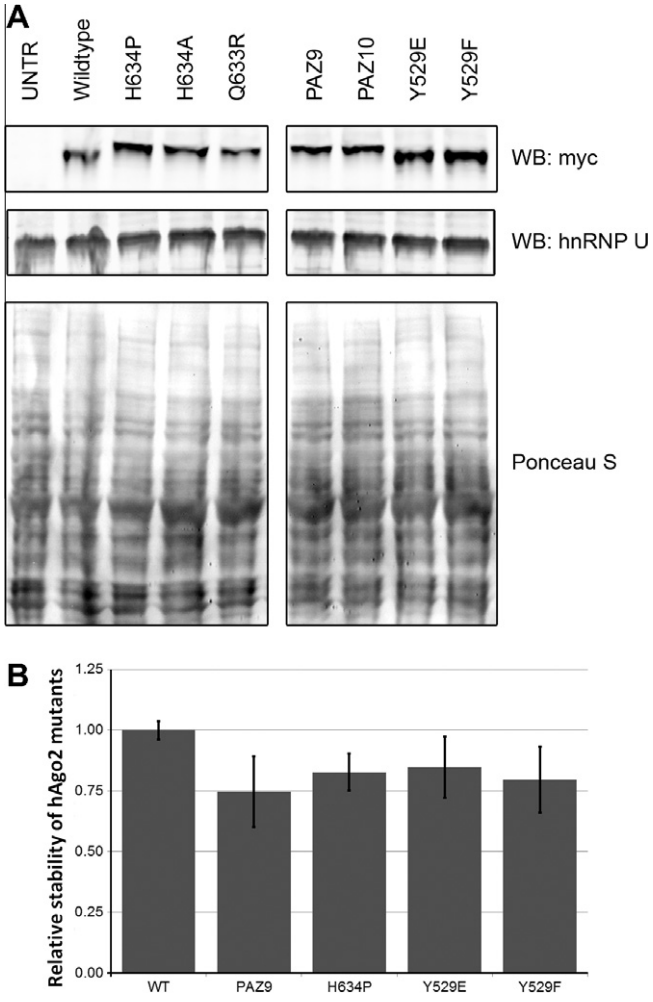

Fig. 3. RNA-binding mutants of hAgo2 are stable. (A) Total cell lysates were prepared from HeLa cells transiently transfected with myc-tagged hAgo2 constructs. The membrane containing the transferred proteins was stained with Ponceau S and then immunoblotted with an antibody against hnRNP U (loading control). Myc-tagged hAgo2 proteins were detected with a mouse monoclonal antibody to myc. (B) HeLa cells, transiently transfected with myc-tagged hAgo2 constructs, were treated with 100 μg/mL cycloheximide for 12 h prior to lysis. Relative levels of hAgo2 proteins were determined by immunoblot analyses. Average values obtained by quantitation of data from three separate experiments were plotted. Error bars represent standard error of the mean.

3.3. RNA-binding mutants of hAgo2 are stable

It was reported that Argonautes that are complexed with small RNAs are much more stable than unloaded Argonaute proteins [35]. Accordingly, it was possible that our failure to detect association of certain hAgo2 mutants with RNA granules was the result of decreased expression, instability of the proteins or proteolytic cleavage of the epitope tag. Therefore, we used immunoblot analyses to compare the relative expression levels of miRNA-binding mutants including PAZ-9, PAZ-10 and Y529E. Following protein transfer, the membranes were stained with Ponceau S and then incubated with an antibody against hnRNP U to show equivalent protein loading (Fig. 3A). Immunoblotting with anti-myc showed that all hAgo2 mutant constructs expressed as well as myc-tagged hAgo2 (Fig. 3A). To determine if the mutants were stable, transfected cells were treated with cycloheximide for 12 h prior to immunoblot analyses. Quantitation of the immunoblots is shown in Fig. 3B. These data indicate that the stabilities of the hAgo2 mutants are similar to that of wild type myc-hAgo2. It should be noted that the wild type myc-hAgo2 was created independently of PAZ-9, PAZ-10, Q633R, H634A and H634P mutants (described in Materials and Methods), while the Y529E and Y529F mutants are derivatives of our wild type hAgo2 construct. The different origins and cloning strategies of these constructs account for the subtle differences in size observed by immunoblotting. As the wild type and PIWI domain mutants (an example of each of the two cloning strategies used) localize to P-bodies and stress granules as expected, it is unlikely that these variations in length could account for any altered localization.

4. Discussion

Argonaute proteins play essential roles in small RNA-dependent gene silencing which affects the expression of most mammalian genes. Accordingly, the activities of Argonaute proteins are subject to extensive regulation. One predicted mechanism for regulating gene silencing is the association of miRNA ribonucleoprotein complexes with cytoplasmic RNA granules. Indeed, the induction of stress granules leads to a decrease in the level of miRNA-mediated silencing [30]; however, the mechanism by which this occurs remains unclear. In fact, very little is known regarding how hAgo2 is targeted to stress granules. Here we report the first integrated analyses of how three different hAgo2 domains function in localization of this protein.

Together, our results confirm and extend the findings of a number of other laboratories, although some results presented here differ significantly from previous reports. For example, using Dicer knock-out cell lines, Leung et al. reported that localization of hAgo2 to stress granules requires the production of miRNAs, but that targeting of hAgo2 to P-bodies does not depend on Dicer activity [27]. Our results suggest that the binding of miRNAs is required for the association of hAgo2 with both P-bodies and stress granules. While our results appear to contradict those of, Leung et al. it is important to point out that some miRNAs do not require Dicer for biogenesis [32,33] and as such, it is quite likely that Dicer knockout cells do in fact contain miRNAs.

Second and somewhat contrary to Johnston et al., who hypothesize that unloaded Argonaute proteins are subject to rapid degradation in the absence of Hsp90 activity, we found that the

stabilities of hAgo2 mutants that are unable to bind small RNAs are not dramatically different from wt hAgo2. The conclusion of Johnston et al. was based on the idea that Hsp90 is required to stabilize unloaded hAgo2. Our previous results are consistent with those of Johnston et al. in that pharmacologically blocking Hsp90 activity results in increased turnover of hAgo2 [36]. However, based on the observation that PAZ and Y529E mutants are in fact quite stable, we conclude that the protective effect of Hsp90 on hAgo2 stability is not directly related to miRNA loading. We also found that, in contrast to Rudel et al., the Y529F mutant is not targeted to P-bodies or stress granules. Some discrepancy may result from differences in quantitation methods. Instead of manually determining the percentage of GW182-positive granules which contained hAgo2, we employed unbiased software-based quantitation to determine the fraction of total hAgo2 colocalized at GW182- or TIA-positive granules. It is also possible that dynamic phosphorylation of Y529 is part of the hAgo2 activity cycle and therefore necessary for RISC loading.

While the significance of Argonaute association with P-bodies and stress granules is still not clear, our results are consistent with the hypothesis that the localization of hAgo2 to RNA granules is mediated by miRNA-dependent interaction with targeted mRNAs. Altering the subcellular localization of Argonaute proteins and their association with RNA granules may serve as an important mechanism for controlling their activities. Accordingly, factors that govern loading of miRNAs onto Argonautes are certain to play key roles in their function within RNAi pathways. Given the broad implications of Argonaute activity on the translation of most human mRNAs, a better understanding of these and other mechanisms for controlling Argonaute activity is surely warranted.

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