

# Function of the Trypanosome Argonaute 1 Protein in RNA Interference Requires the N-terminal RGG Domain and Arginine 735 in the Piwi Domain\*<sup>§</sup>

Received for publication, August 12, 2004, and in revised form, September 20, 2004  
Published, JBC Papers in Press, September 21, 2004, DOI 10.1074/jbc.M409280200

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**Argonaute proteins are central components of RNA interference (RNAi) and related phenomena in a wide variety of eukaryotes, including the early diverging protozoan *Trypanosoma brucei*. The single *T. brucei* Argonaute protein (TbAGO1) is in a complex with small interfering RNAs (siRNAs), and a fraction of this ribonucleoprotein particle is associated with polyribosomes. In this study, we generated a panel of insertion, deletion, and single point mutants of TbAGO1 and assayed them *in vivo* for their function in RNAi. In addition to the signature domains of Argonaute proteins, PAZ and Piwi, TbAGO1 has an N-terminal domain with a high abundance of RGG repeats. Deletion of the N-terminal domain blocked association of AGO1 with polyribosomes and severely affected mRNA cleavage. Nevertheless, the mutant protein was in a complex with siRNAs. In contrast, deletion of the Piwi domain led to a loss of siRNAs but did not abolish polyribosome association. Site-directed mutagenesis of conserved amino acids in the Piwi domain identified arginine 735 as essential for RNAi. Although the R735A mutant bound siRNAs and associated with polyribosomes, it displayed a severe defect in the cleavage of target mRNA.**

small interfering RNAs (siRNAs) or microRNAs, which are produced as a result of cleaving dsRNA by an endonuclease termed Dicer. Second, they share the incorporation of these small RNAs into a multimeric ribonucleoprotein complex, known as RNA-induced silencing complex, containing at least one member of the Argonaute (AGO) protein family.

The Argonaute family of proteins was first defined in *Arabidopsis thaliana* by way of a genetic screen (2) and was subsequently linked to RNAi through biochemical studies in *Drosophila* (3). In single cell eukaryotes there appear to be one or two AGO family genes, whereas in multicellular organisms this gene family has expanded to include up to 24 family members as in the case of *Caenorhabditis elegans* (4). Argonaute proteins are characterized by two domains: a PAZ domain of ~110 residues found near the middle of the protein and a C-terminal Piwi domain (4). Structural studies have defined the PAZ domain as an RNA-binding module (5–7), and this domain might function as an anchoring site for the two-nucleotide 3'-overhang of siRNAs (8). The 300-amino acid Piwi domain displays a high degree of similarity among Argonaute family members and is present in prokaryotes (9). Very recent structural studies (10, 11) have revealed that Piwi is similar to an RNase H domain, thus supporting a model for Argonaute as the nuclease responsible for targeted mRNA cleavage.

Here we have undertaken a functional analysis of the *T. brucei* Argonaute 1 (TbAGO1) protein, the only member of the Argonaute family identified in this organism. Previous studies (12) have demonstrated that TbAGO1 is essential for RNAi and is required for accumulation of siRNAs and for down-regulation of endogenous retroposon transcript levels. Furthermore, a proportion of a ribonucleoprotein complex containing TbAGO1 and siRNAs is found associated with polyribosomes, suggesting a connection between the RNAi and translation machinery in trypanosomes (12, 13). In the present study, we built on the observation that AGO1 knock-out cells in *T. brucei* are viable and have tested mutations in AGO1 for their ability to restore RNAi in this genetic background.

RNA interference (RNAi)<sup>1</sup> is a gene-silencing mechanism that is triggered by double-stranded RNA (dsRNA). RNAi is operational in a large number of eukaryotic organisms, including the early diverging protozoan *Trypanosoma brucei*, suggesting an ancient evolutionary origin. In recent years, RNAi involvement has been discovered in a variety of phenomena that effect gene silencing at the RNA or DNA level. These include mRNA degradation, translational or transcriptional repression, histone and DNA methylation, and programmed DNA elimination (reviewed in Ref. 1). Although on the surface these gene-silencing phenomena might appear quite different, they so far share two characteristics. First, they share the presence of ~20–26-nucleotide-long small RNAs, referred to as

## EXPERIMENTAL PROCEDURES

**Construction of AGO1 Mutants**—To generate random insertions in TbAGO1, we used the *EZ::TN* in-frame linker insertion kit (EPICENTRE). To favor insertions in the *TbAgo1* gene, we subcloned the *XhoI*-*EcoRI* fragment containing the open reading frame and the 5'-untranslated region into the pSP72 vector. The transposition reaction was carried out *in vitro* according to the manufacturer's instructions. The DNA was transformed into *Escherichia coli* cells, and PCR screening of bacterial colonies with transposon- and *Ago1*-specific primers was used to identify insertions in the *Ago1* gene. The kanamycin resistance gene was removed by *NotI* digestion, leaving a 57-bp (19 amino acids) in-frame insertion. 10 insertions scattered throughout the *Ago1* gene were transferred into the complementation vector and transformed into *ago1*<sup>-/-</sup> cells as described previously (12).

\* This work was supported by National Institutes of Health Grant AI28798 (to E. U.). 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.

<sup>§</sup> The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. 1.

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<sup>1</sup> The abbreviations used are: RNAi, RNA interference; dsRNA, double-stranded RNA; siRNAs, small interfering RNAs; AGO, Argonaute protein; TbAGO, *T. brucei* AGO; siRNP, small interfering ribonucleoprotein; Tn, transposon.

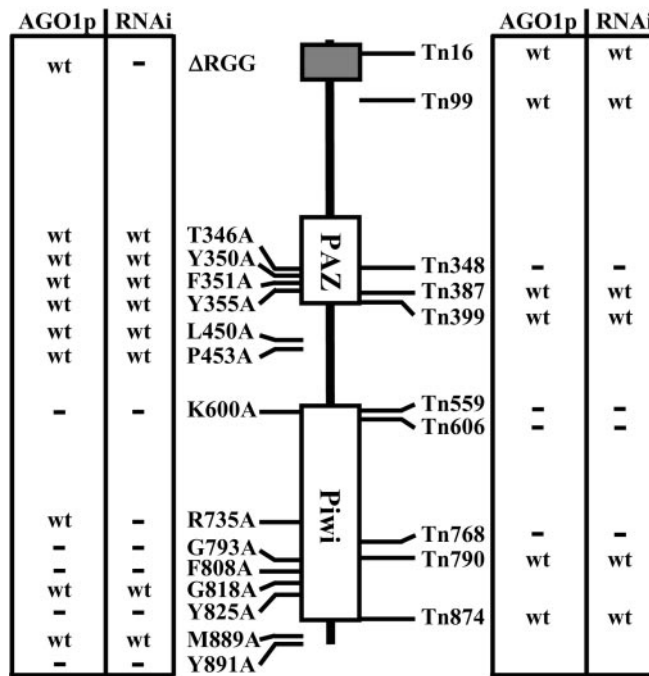


FIG. 1. Summary of *T. brucei* AGO1 mutants. The central part of the figure depicts a schematic representation of the Tn5 insertion mutants on the right and the single amino acid substitutions on the left. Protein levels were determined by Western blot with anti-AGO1 antibodies (AGO1p) and listed as being similar (wt) or greatly reduced (-) compared with wild-type levels. The RNAi phenotype was assayed by transfecting  $\alpha$ -tubulin dsRNA and monitoring the FAT phenotype as described (22). wt, RNAi was restored to wild-type levels; -, no restoration of RNAi. Note that  $\Delta$ RGG and R735A have residual RNAi activity as described in the legend for Fig. 5. The N-terminal RGG domain is indicated as a filled box.

Deletion constructs were generated by PCR and cloned into the complementation vector. Deletion of the RGG domain ( $\Delta$ RGG) removed amino acids 2–68. In  $\Delta$ 2–267, amino acids 2–267 of AGO1 were removed.  $\Delta$ 2–418 did not contain amino acids 2–418, and  $\Delta$ Piwi was missing amino acids 550–903. Epitope tags in TbAGO1 and single amino acid substitutions in the AGO1 protein were introduced by a two-step PCR procedure, and all mutant constructs were verified by DNA sequencing.

**Other Procedures**—RNA extraction, dsRNA transfection, and Northern blot analysis were performed as described previously (14). Immunoprecipitations and Western blot analysis were performed as described previously (see Ref. 12). Cell fractionation and sucrose density gradient analysis of cytoplasmic extracts were carried out as described previously (13).

## RESULTS

**Random In-frame Linker Insertions in AGO1**—In a first approach to localizing functional domains in the *T. brucei* AGO1 protein, we generated several hundred random 57-bp (19 amino acids) in-frame insertions using a transposon-based strategy. To assess the validity of this strategy, we arbitrarily selected 10 insertions scattered across the target gene (Fig. 1 and supplemental Fig. 1) and tested them *in vivo* by complementation of *ago1*<sup>-/-</sup> cells as described previously (12). Next, cells expressing the various AGO1 insertion mutants were examined for RNAi competency by assaying the ability of dsRNA corresponding to the  $\alpha$ -tubulin mRNA to produce the FAT phenotype (cells with multiple nuclei, flagella, and mitochondrial genomes). As reported previously (12), *ago1*<sup>-/-</sup> cells are deficient in RNAi (*i.e.* no FAT cells), and complementation with the wild-type AGO1 gene restored RNAi. In 6 of the 10 insertions tested, the FAT phenotype was apparent following electroporation with  $\alpha$ -tubulin dsRNA (Fig. 1), revealing that these particular alterations in the RGG, PAZ, and Piwi do-

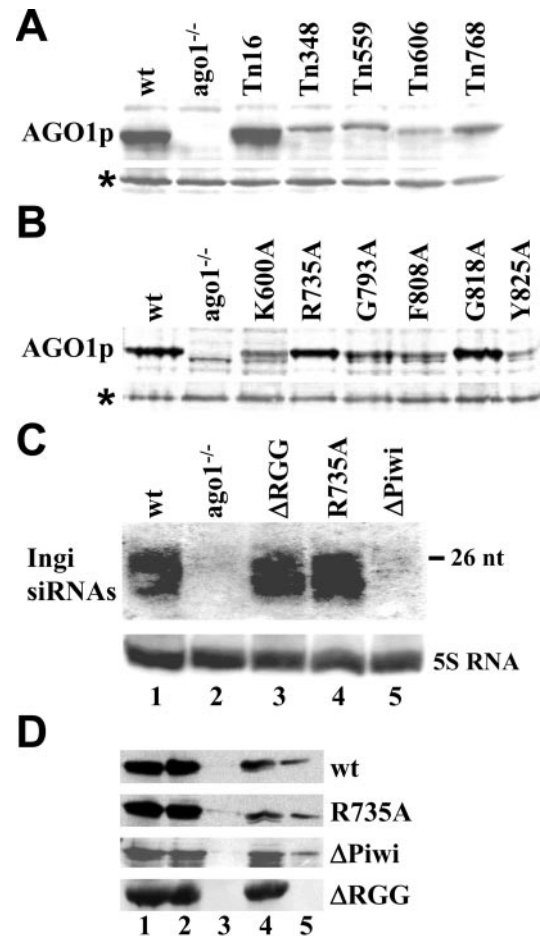


FIG. 2. Analysis of mutant AGO1 proteins. A selected number of cell lines expressing either AGO1 proteins with 19-amino acid in-frame insertions (A) or single amino acid substitutions (B) were analyzed by Western blot with anti-AGO1 antibodies. An immunologically cross-reacting protein was used as a loading control (\*). C, the level of Ingi siRNAs was probed by Northern blot in wild-type (wt) cells (lane 1), AGO1-deficient (*ago1*<sup>-/-</sup>) cells (lane 2), and cells expressing  $\Delta$ RGG (lane 3), R735A (lane 4), or  $\Delta$ Piwi (lane 5). A DNA size marker in nucleotides (nt) is indicated on the right. The hybridization to 5 S rRNA (5S rRNA) served as a loading control. D, Western blot is shown with anti-AGO1 polyclonal antibodies of equivalent amounts of total extract (lane 1), post-nuclear pellet (lane 2), and supernatant (lane 3), S100 fraction (lane 4), and ribosomal pellet (lane 5) of cells expressing wild-type, R735A,  $\Delta$ Piwi, or  $\Delta$ RGG AGO1 proteins.

mainly did not affect AGO1 function. On the other hand, four insertional mutations failed to re-establish RNAi; one was in the PAZ domain, and the other three were in the Piwi domain.

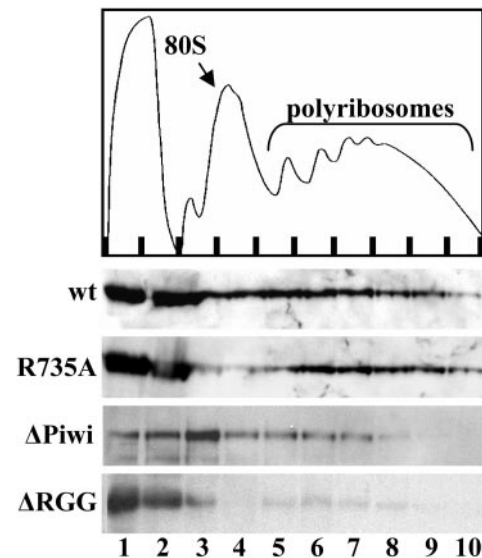
The lack of complementation by four constructs might reflect a loss of function and/or instability of the mutant protein. Thus, we determined the steady-state levels of the mutant proteins by Western blot analysis with anti-AGO1 antibodies (Fig. 2A). There was no significant difference in the level of wild-type protein and mutant AGO1 proteins capable of restoring RNAi. However, the four non-functional AGO1 proteins either were not detected or were present at greatly reduced levels, suggesting that the proteins were unstable. This was supported by Northern blot analysis, which showed equal accumulation of mRNA in all cell lines (data not shown). Thus, the phenotypes of the mutants accumulating low amounts of the AGO protein are difficult to interpret in terms of their activity in RNAi.

**Deletion Constructs and Targeted Point Mutations of Highly Conserved Residues in AGO1**—To further map the functional domains in TbAGO1, we generated four deletion constructs, namely,  $\Delta$ RGG (deletion of residues 2–61),  $\Delta$ 2–267 (deletion of

the N terminus up to the PAZ domain),  $\Delta 2-418$  (deletion of the N terminus including the PAZ domain), and  $\Delta$ Piwi (deletion of the Piwi domain at the C terminus). Furthermore, by means of the multiple sequence alignment programs ClustalW and T-Coffee, we constructed 14 single amino acid substitutions throughout the protein, using as guidance the results of the alignment of the TbAGO1 sequence with other members of the Argonaute family. Originally, 22 Argonaute proteins were included in the analysis, but only a representative subset is shown in supplemental Fig. 1. As noted previously, the highest conservation among the different proteins was observed in the Piwi domain (9), which in TbAGO1 covers amino acids 553–873. In this region, six residues are highlighted that are 100% conserved among the different members of the Argonaute family. In contrast, in the second signature motif, the PAZ domain (amino acids 279–405 in TbAGO1), there are no residues that are strictly conserved. Other than these two domains, the sequence conservation among all the Argonaute proteins is rather limited. Nevertheless, one noteworthy result of these alignments was the identification of a conserved short sequence motif in the last four amino acids at the C terminus, (M/L)X(F/Y)X. Interestingly, one of the RNAi-deficient mutant alleles of *C. elegans* RDE-1, *ne297*, maps to a glycine residue immediately upstream from the conserved motif (supplemental Fig. 1 and Ref. 15).

Based on the above alignments, the six residues strictly conserved in the Piwi domain were selected for site-directed mutagenesis studies and individually substituted by alanine (Fig. 1 and supplemental Fig. 1). In addition, we mutated four residues in the PAZ domain and two residues in the region between the PAZ and Piwi domains that are conserved among a subset of Argonaute proteins. Finally, we targeted the methionine and tyrosine residues at the very C terminus.

**Summary of Phenotypic Analysis of AGO1 Mutations**—Following the establishment of stable cell lines in the *ago1*<sup>-/-</sup> background, the 14 single amino acid changes in the AGO1 protein and the deletion mutants were scored for the restoration of the RNAi phenotype by assaying the ability of dsRNA corresponding to the  $\alpha$ -tubulin gene to produce the FAT phenotype and for the level of protein expression. Based on these results, the mutants were placed into three categories (Fig. 1). The first group of mutants (T346A, Y350A, F351A, Y355A, L450A, P453A, G818A, and M889A) had no effect on RNAi, *i.e.* complementation of the *ago1*<sup>-/-</sup> cells restored the RNAi phenotype to wild-type levels (data not shown). In addition, Western blot analysis revealed that these mutant proteins had expression levels comparable with that of wild-type AGO1 (Fig. 2B) (data not shown). In contrast, the second set of mutants, including the single amino acid changes K600A, G793A, F808A, Y825A, and Y891A and the deletion constructs  $\Delta 2-267$  and  $\Delta 2-418$ , displayed extremely poor expression levels of the AGO1 protein and were therefore not included in further experiments (Fig. 2B) (data not shown). It was somewhat unexpected to find that five of the eight mutants made in the Piwi domain and in the conserved C-terminal motif accumulated very little protein. Because the mRNA levels of these five mutants were comparable with wild-type levels (data not shown), this might suggest that the Piwi domain structure is very sensitive to perturbations and easily misfolds. Alternatively, it might suggest that the Piwi domain might be required for interaction with another component of the RNAi pathway and that the mutations prevent this functional interaction from occurring and as a consequence the AGO1 protein is degraded. Finally, the  $\Delta$ RGG and  $\Delta$ Piwi mutants as well as one single point mutation in the Piwi domain (R735A) did not restore RNAi but had protein expression levels comparable with that of

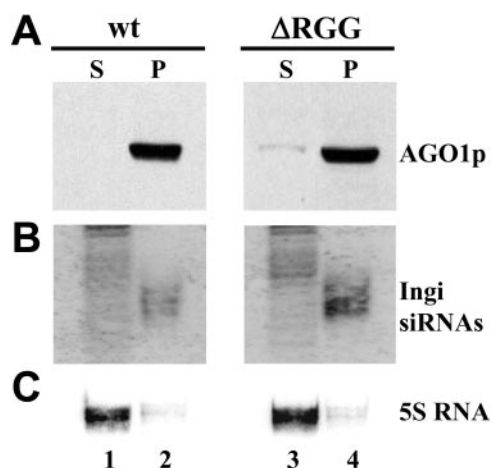


**FIG. 3. Sucrose density gradients of cytoplasmic extracts from cells expressing wild-type (*wt*), R735A,  $\Delta$ Piwi, or  $\Delta$ RGG AGO1.** The upper panel shows a representative absorbance profile at 254 nm, and the positions of the 80 S monosome and polyribosomes are indicated. The four panels below the absorbance profile show a Western blot analysis of the sucrose density gradient fractions using a rabbit anti-AGO1 polyclonal antibody.

wild-type AGO1 (Fig. 2D) and thus were subjected to a more detailed analysis.

**R735A and the  $\Delta$ RGG Affect the Effector Step of RNAi**—To examine what step of the RNAi pathway was affected by the R735A and the two deletion mutants ( $\Delta$ RGG and  $\Delta$ Piwi), we first monitored the accumulation of endogenous siRNAs derived from *Ingi* retroposon transcripts. Whereas *ago1*<sup>-/-</sup> cells are deficient in the accumulation of siRNAs derived from *Ingi* transcripts (Fig. 2C, lane 2) (12), the abundance of *Ingi* siRNAs was not noticeably affected by either the R735A or  $\Delta$ RGG mutation (Fig. 2C, lanes 3 and 4). In contrast, deletion of the Piwi domain manifested a phenotype similar to *ago1*<sup>-/-</sup> cells, with no apparent accumulation of *Ingi* siRNAs (Fig. 2C, lane 5).

Next, we analyzed the distribution of the R735A,  $\Delta$ Piwi, and  $\Delta$ RGG mutant proteins in cell extracts by differential centrifugation (Fig. 2D). As we have shown previously (12), wild-type AGO1 is mostly found in the postnuclear supernatant (Fig. 2D, lane 2) and in the S100 fraction (Fig. 2D, lane 4). In addition, a proportion of AGO1 is present in the 100,000  $\times$  *g* pellet together with large complexes, including ribosomes (Fig. 2D, lane 5). A similar analysis of the R735A and  $\Delta$ Piwi mutants revealed fractionation properties comparable with those of wild-type AGO1 (Fig. 2D). In contrast, centrifugation of a cell extract from the  $\Delta$ RGG mutant at 100,000  $\times$  *g* revealed that the protein was exclusively recovered in the supernatant, with no detectable levels in the pellet (Fig. 2D, compare lanes 4 and 5). To further evaluate the sedimentation characteristics of the mutant AGO1 proteins, cytoplasmic extracts were centrifuged through a 15–50% sucrose gradient, and individual fractions were analyzed by Western blotting with anti-AGO1 antibodies (Fig. 3). By this analysis, R735A and  $\Delta$ Piwi partially co-sedimented with polyribosomes, similar to wild-type AGO1 protein. However, it is worth noting that the deletion of the Piwi domain changed the sedimentation behavior of the Argonaute protein in that the majority of the protein fractionated with an apparent sedimentation value of 40–60 S. At present, we have no explanation for this observation, but experiments are in progress to address this issue. In contrast, the majority of the  $\Delta$ RGG mutant protein was found near the top of the gradient, and there was very little of the protein in the gradient fractions



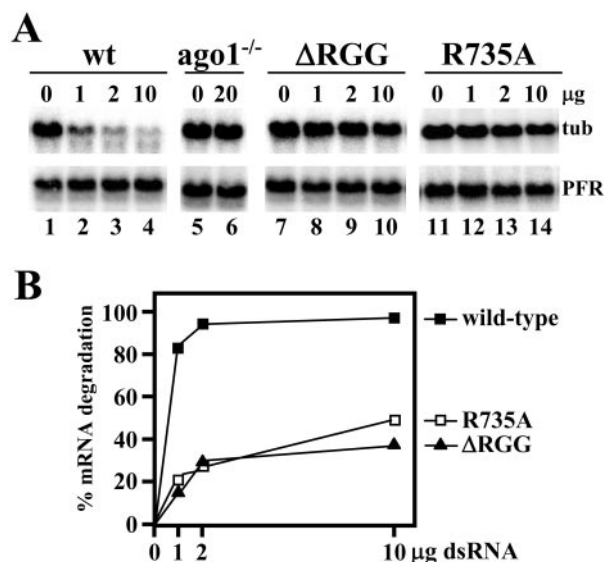
**FIG. 4. Association of wild-type (*wt*) and  $\Delta$ RGG AGO1 with Ingi siRNAs.** A cytoplasmic extract from cells expressing N-terminal BB2-tagged AGO1 or N-terminal BB2-tagged  $\Delta$ RGG was subjected to immunoprecipitation with anti-BB2 antibodies, and supernatant (S) and pellet (P) fractions were processed for Western blot analysis for AGO1 (A) and Northern blot analysis for Ingi siRNAs (B) as well as for 5S RNA to control for immunoprecipitation specificity (C).

where polyribosomes sediment (Fig. 3). One possible explanation for this result is that although siRNAs were present in the  $\Delta$ RGG background, they did not form a ribonucleoprotein complex with the mutated AGO1 protein. To address this issue, an epitope-tagged version of the  $\Delta$ RGG protein was immunoprecipitated from a cytoplasmic extract, and the immunoprecipitate was assayed by Northern blotting for the presence of Ingi siRNAs (Fig. 4). This assay revealed that the deletion of the RGG domain does not impair the formation of AGO1-siRNA complexes. Thus, it appeared that the N-terminal RGG domain contains a determinant for the association of AGO1 with polyribosomes.

Because cells expressing the  $\Delta$ RGG or R735A mutant proteins accumulated wild-type levels of siRNAs, we next tested whether they are competent to cleave target mRNA on exposure to trigger dsRNA. For this test, cells expressing wild-type,  $\Delta$ RGG, and R735A AGO1 were electroporated with different amounts of  $\alpha$ -tubulin dsRNA, and the fate of the targeted mRNA was monitored by Northern blot analysis (Fig. 5A). As expected, wild-type cells were very sensitive to dsRNA, with 1  $\mu$ g resulting in the degradation of more than 80% of  $\alpha$ -tubulin mRNA (Fig. 5A, lane 2). With higher doses of dsRNA, the extent of degradation of  $\alpha$ -tubulin mRNA reached a plateau, indicating that the reaction was saturated. In contrast, at all dsRNA doses tested, cells expressing  $\Delta$ RGG or R735A AGO1 demonstrated a severe reduction in their ability to target  $\alpha$ -tubulin mRNA for degradation (Fig. 5B). In particular, using 1  $\mu$ g of dsRNA/transfection, the efficiency of the last step of RNAi was reduced by approximately 4-fold in both mutant backgrounds. In both cases, at doses of dsRNA that are saturating for wild-type cells, there was only a slight increase in the degradation of  $\alpha$ -tubulin mRNA. Thus,  $\Delta$ RGG and R735A proteins are severely defective in the efficiency of cleavage of target mRNA, namely, in the effector step of RNAi.

#### DISCUSSION

Our goal in this study was to provide a better understanding of the molecular function of Argonaute 1 in the RNAi pathway in *T. brucei*. To do this, we used *in vivo* complementation of an *ago1*<sup>-/-</sup> strain as a means to test the functional significance of predicted domains in AGO1. Like other members of the Argonaute family, the *T. brucei* protein contains a centrally located PAZ domain and a C-terminal Piwi domain. An additional



**FIG. 5. Assay for RNAi.** A, wild-type (*wt*) cells (lanes 1–4), AGO1 knock-out (*ago1*<sup>-/-</sup>) cells (lanes 5 and 6), and  $\Delta$ RGG (lanes 7–10) and R735A cells (lanes 11–14) were challenged with poly(dI-dC) (lanes 1, 5, 7, and 11) or different amounts ( $\mu$ g) of  $\alpha$ -tubulin (*tub*) dsRNA as indicated above each lane. The level of  $\alpha$ -tubulin mRNA was monitored by Northern blot (upper panel), and paraflagellar rod (*PFR*) mRNA served as a control for RNA recovery and loading (lower panel). B, quantitation of the results shown in A.

noticeable feature of AGO1 is the presence at the N terminus of five direct repeats that are rich in arginine and glycine residues with a high abundance of RGG repeats. We have previously hypothesized that this RGG domain is subjected to post-translational modifications by an arginine methyltransferase (12).

In one series of mutant proteins, we randomly inserted 19 amino acids by a Tn5-based transposon strategy and tested 10 of them for their function in RNAi. Whereas four proteins were inactive and were detected at much reduced levels in cells, we were surprised to find six insertions that were tolerated at sites distributed throughout the protein, including the RGG, PAZ, and Piwi domains. Of particular interest are the three insertions in the PAZ domain, because its structure has recently been determined in the free state (5–7) as well as in a complex with a 9-mer siRNA-like duplex (8). The two permissive sites (Fig. 1, *Tn387* and *Tn399*) can be reconciled with being near the C terminus of the PAZ fold, in a region that appears to be unstructured. On the other hand, insertion *Tn348* was not tolerated and is located in an aromatic cluster forming helix  $\alpha$ 3, which lines the RNA-binding pocket of the PAZ domain. Thus, it is very likely that the insertion of 19 amino acids in this functionally important region of the PAZ domain resulted in a severe defect in protein folding and/or stability, which is manifested by very low levels of protein accumulation in cells. However, it is somewhat intriguing that when we probed individual conserved residues in this helix by site-directed mutagenesis, namely Thr-346, Tyr-350, Phe-351, and Tyr-355, we did not observe an effect on protein expression or RNAi function (Fig. 1). Because the primary sequence of the TbAGO1 PAZ domain is quite divergent from that of higher eukaryotes and cannot be convincingly modeled on the existing PAZ domain structure, structural studies will be required to interpret the effect of the above mentioned mutations.

One intriguing aspect of RNAi, although not fully understood, is the potential interplay with translation. In particular, evidence is accumulating to support a functional link between RNA-induced silencing complexes or RNA-induced silencing complex components and ribosomes (3, 12, 13, 16–19). In the present study, we have shown that the N-terminal RGG do-

main of TbAGO1 is required for the stable association of the AGO1-siRNP with polyribosomes. At the same time, deletion of this domain severely impairs but does not completely abolish cleavage of target mRNA. It is tempting to speculate that the association of the AGO1-siRNP with polyribosomes increases the efficiency of target mRNA cleavage, at least in the case of those mRNAs that, like  $\alpha$ -tubulin, are almost quantitatively associated with polyribosomes (20). The residual RNAi activity of the  $\Delta$ RGG protein might be taken as an indication that mRNA cleavage can occur in the absence of an association of the AGO1-siRNP with polyribosomes. Alternatively, it is possible the  $\Delta$ RGG-siRNP might still bind to translating ribosomes but with decreased affinity relative to the wild-type particle.

The RGG domain of AGO1 might bind to ribosomes directly or indirectly via interaction with other components. Indeed, RGG motifs appear to be bifunctional modules for RNA binding and/or for protein-protein interactions, and they are known targets for methylation of arginine residues by type I or type II protein arginine methyltransferase (21). It has been argued that the addition of methyl groups to arginine might serve as a means to modulate either intra- or intermolecular protein-protein interactions. At present we do not know whether RNA binding and/or arginine modifications of the AGO1 RGG domain are involved in the observed polyribosome association. However, the preliminary findings from mass spectrometry examination of TbAGO1 are consistent with the presence of dimethylated arginine residues in the RGG domain,<sup>2</sup> and we are currently investigating the role of arginine methylation in the activity of TbAGO1 in RNAi. Finally, it is important to point out that the N-terminal RGG domain is not a unique feature of the trypanosome Argonaute protein. Similar repeats, although not as prevalent, are found at the N terminus of *Arabidopsis* AGO1.

We hypothesized previously (13) that siRNPs interact directly with one or more components of the ribosome, similarly to what has been described in *Drosophila* (18). Furthermore, we argued that this association is not mediated by the interaction of siRNAs with their specific target mRNA and is most likely independent of the identity of the mRNA that is being translated. One of our deletion mutants corroborates this hypothesis in that the removal of the Piwi domain generated a scenario in which siRNAs did not accumulate and hence did not form a complex with the mutated AGO1. Nevertheless, this protein associates with polyribosomes, underscoring that siRNAs do not play a role in this interaction.

In a second approach to identifying essential residues for RNAi function, we produced a panel of 14 site-directed AGO1 mutants. Remarkably, we found that five of eight mutants introduced in the Piwi domain and at the very C terminus had phenotypes similar to the non-functional insertion mutants described above, consisting of very low intracellular accumulation of the Argonaute protein and an inability to complement the RNAi deficiency. Nevertheless, our screen generated one mutant protein (R735A) that was expressed at levels similar to

wild-type AGO1 but failed to restore RNAi. Arg-735 in the Piwi domain is strictly conserved in all members of the Argonaute gene family in eukaryotes but is not present in the few available Piwi-containing proteins in prokaryotes. Our detailed phenotypic analysis revealed that cells expressing the R735A mutant were defective in RNAi at the step of target mRNA cleavage, suggesting that the Piwi domain was responsible for mRNA cleavage, either directly or indirectly. While this manuscript was in preparation, two reports (10, 11) appeared that put forward a model for mammalian Argonaute 2 as the nuclease in charge of mRNA cleavage. In particular, structural studies of a *Pyrococcus furiosus* Argonaute protein revealed the Piwi domain to be a member of the RNase H family, with an active site triad of acidic residues termed the DDE motif. Remarkably, a fourth residue in the center of the active site is an arginine and corresponds to arginine 735 in the *T. brucei* protein. Taken together, we can put forward the notion that mutating Arg-735 to alanine inactivated the catalytic activity of the Piwi domain, which by analogy to mammalian Argonaute 2 would be cleavage of the target mRNA. Thus, it appears very likely that the *T. brucei* Argonaute 1 protein functions as the nuclease in RNAi.

*Acknowledgment*—We thank Shulamit Michaeli for helpful comments on the manuscript. We also thank the class of the Biology of Parasitism course, 2002, for their help in this project.

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<sup>2</sup> N. Chamond, A. Djikeng, E. Ullu, and C. Tschudi, unpublished observation.