Selection and Characterization of RNA Interference-Deficient Trypanosomes Impaired in Target mRNA Degradation

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Genetic analysis of the RNA interference (RNAi) pathway in Trypanosoma brucei has so far revealed one essential component, namely, TbAGO1, encoding a member of the Argonaute protein family. To gain further insight into the RNAi mechanism and its biological significance, we selected RNAi-deficient trypanosomes by using repeated cycles of electroporation with α-tubulin double-stranded RNA, a treatment that blocks cytokinesis in wild-type cells. Two independent clones, termed RID-1 (for RNAi-deficient clone 1) and RID-2, were characterized. At the cellular level, only RID-1 trypanosomes showed a significant increase in doubling time with the concomitant accumulation of cells defective in the completion of cytokinesis. At the RNA level, both clones accumulated wild-type amounts of small interfering RNAs and displayed elevated levels of retroposon transcripts, the hallmark of RNAi deficiency in T. brucei. Importantly, both RID-1 and RID-2 clones were defective in the degradation of target mRNA, suggesting an impairment of the activity of AGO1, the putative RNAi endonuclease. Since in RID cells the AGO1 gene was not mutated and was expressed at wild-type levels, we propose that in trypanosomes the cleavage of mRNA by AGO1 is regulated by the interaction with another factor(s).

RNA interference (RNAi) refers to a posttranscriptional gene-silencing mechanism that is triggered in many eukaryotic organisms by double-stranded RNA (dsRNA). Early on it was recognized that one of the biological functions of RNAi is to destroy transcripts derived from molecular parasites, namely, viruses, transposons, retroposons, or transgenes. However, in the course of the last few years it has become evident that the RNAi pathway is also implicated in a variety of gene-silencing phenomena as diverse as heterochromatin assembly (29) and RNAi pathway: the accumulation of transgene- or retroposon-derived siRNAs is drastically reduced, most likely because association with AGO1 is required to stabilize siRNAs, and target mRNAs are not degraded (21). These studies also showed that
**MATERIALS AND METHODS**

**Growth and manipulation of cells.** YTat 1.1 procyclics were maintained at 27°C as previously described (21). Selection of cells resistant to α-tubulin dsRNA challenge was performed as follows. Trypanosomes (10^9) were divided into 10 aliquots, and each aliquot was electroporated with 10 μg of synthetic α-tubulin dsRNA corresponding to the 5′ untranslated region of α-tubulin mRNA as previously described (19) and then returned to culture for expression of the FAT phenotype. After approximately 48 h of growth, the cells in the medium were removed from the culture, taking care not to disturb the cells at the bottom of the flask, which consisted mainly of large aggregates of FAT cells. The surviving cells were collected by centrifugation, resuspended in 10 ml of fresh medium, and allowed to grow until they reached a cell density of about 10^7/ml. At this point a second round of electroporation and growth was performed, and this procedure was repeated a total of five times. Clonal RD-1 and RD-2 cell lines were then established by limited dilution in microtiter dishes.

**Nucleic acid and protein analyses.** Northern and Western blot analyses were performed as previously described (21). For expression of green fluorescent protein (GFP) dsRNA, cells were transformed with a plasmid construct expressing hairpin GFP dsRNA as previously described (21). Synthesis of radiolabeled RNA in permeable cells was carried out as previously described (24).

**Cytological and immunofluorescence assays.** For determination of the cell cycle status, trypanosomes were spread onto microscope slides and air dried. The slides were fixed for at least 30 min in 100% methanol at −20°C and stained with DAPI (4,6-diamidino-2-phenylindole). At least 500 cells were counted for each cell line, and three independent cell counts were carried out on different, independently grown cell populations. The flagellum was stained with a monoclonal mouse antibody directed to the paraflagellar rod protein (PFR) (19), followed by 1 μg of Alexa Fluor 566-conjugated goat anti-mouse antibody [goat anti-mouse immunoglobulin G (heavy plus light chain)] F(ab')2; fragment conjugate from Molecular Probes] per ml.

**FACS analysis.** Cells were resuspended at a density of 2 × 10^6/ml in 70% ice-cold ethanol and fixed overnight at 4°C. The cell pellet was resuspended in phosphate-buffered saline containing 1 mg of glucose per ml, 10 μg of propidium iodide per ml, and 10 μg of RNase A per ml and incubated at room temperature for 30 min. Fluorescence-activated cell sorting (FACS) was performed with a Becton Dickinson FACSCalibur with detector FL2-A and an AmpGain of 1.85. Data were analyzed by using the software CellQuest version 5.3.

**RESULTS**

**Selection and characterization of trypanosomes resistant to challenge with α-tubulin dsRNA.** One of the properties of electroporation of synthetic dsRNA in *T. brucei* is its high efficiency, which routinely approaches 90 to 98%. In particular, we have previously shown that transfection of trypanosomes with α-tubulin dsRNA results in degradation of target mRNA and transient downregulation of α-tubulin synthesis (19). At the cellular level cytokinesis is blocked, but the cells continue to duplicate various organelles, lose cytoskeleton integrity, and increase in volume (FAT phenotype). FAT cells form large aggregates and eventually die. This observation provided a powerful and simple approach to enrich for trypanosomes that are defective in the acquisition of the FAT phenotype (Fig. 1). We reasoned that among the cells unable to become FAT in response to electroporation of α-tubulin dsRNA, we might recover trypanosomes that were defective at some step of the RNAi pathway. In a pilot experiment, 10^9 insect-form (procyclic) *T. brucei rhodesiense* YTat1.1 cells, without prior mutagenesis, were electroporated with saturating amounts of α-tubulin dsRNA and allowed to grow in order to develop the FAT phenotype. Next, the cells that had not acquired the FAT phenotype, which represented 2 to 5% of the initial cell population, were separated from FAT cells and expanded (see Materials and Methods for details). This constituted one selection cycle. We performed five successive rounds of electroporation and growth, and after the fifth round more than 90% of the cells did not acquire the FAT phenotype upon challenge with α-tubulin dsRNA. To exclude the possibility that the α-tubulin dsRNA-resistant cells had become resistant to taking up nucleic acids by electroporation, we measured the efficiency of transient expression of a plasmid expressing GFP. The result of this test showed no significant difference in transfection efficiency between wild-type and α-tubulin dsRNA-resistant cells (data not shown). Next, we transfected into the α-tubulin dsRNA-resistant cells a plasmid engineered for high levels of expression of α-tubulin dsRNA as a hairpin (19). Even when the dsRNA was produced inside the cells, the α-tubulin dsRNA-resistant cells did not acquire the FAT phenotype (data not shown). This test eliminated the possibility of defects in the uptake of dsRNA. On the basis of these observations we concluded that the α-tubulin dsRNA-resistant cells were likely to be impaired in responding to α-tubulin dsRNA, possibly because they were defective at some step of RNAi. Northern blot analysis of the fate of α-tubulin or actin mRNA following
transfection of the corresponding dsRNA confirmed this possibility (data not shown, but see Fig. 4).

Having established a proof of principle, we decided to test whether the genetic background of the starting trypanosome population affected the selection process. Indeed, our laboratory strain, YTat1.1, had been propagated in culture for several years, and it was likely that its genetic constitution was heterogeneous. Thus, we derived two clonal cell lines, termed Y1 and Y2, from the YTat1.1 trypanosome population and subjected each of them to the selection procedure described above. As observed before, after five cycles of electroporation and growth more than 90% of the cells failed to respond to challenge with α-tubulin dsRNA. Next, to test whether acquisition of resistance to α-tubulin dsRNA was a specific property of our *T. brucei* laboratory strain, the same selection procedure was applied to two other *T. brucei* laboratory strains. These were 29.13 procycls (a derivative of *T. brucei* 427), which are commonly used for establishing tetracycline-inducible expression (31), and *T. brucei brucei* 927 procycls, the trypanosome strain used for genome sequencing (26). In both cases, cells resistant to α-tubulin dsRNA could be obtained (data not shown). However, it required seven to eight selection cycles for the 29.13 and 927 trypanosomes to become more than 90% resistant to α-tubulin dsRNA, compared to five cycles as we observed with the uncloned YTat1.1 strain or the clonal derivatives Y1 and Y2.

The α-tubulin dsRNA-resistant trypanosomes derived from selection of the Y1 and Y2 cell lines were then cloned by limited dilution. One clone from each of the two populations, namely Y1-13 and Y2-11, were selected as being representative of several that showed the least response to α-tubulin dsRNA. Henceforth these clones will be referred to as RiD-1 (for RNAi-deficient clone 1) and RiD-2.

**Cell cycle and cytological analysis of RiD trypanosomes.** During the course of our analyses, we observed that RiD-1 trypanosomes were growing more slowly than wild-type and RiD-2 trypanosomes. To precisely determine the extent of the growth defect, we monitored cell growth rates in the following way. Cells were seeded at 5 × 10⁵/ml, allowed to grow for 24 h, counted, and diluted again to 5 × 10⁵/ml. This procedure was repeated six times. Figure 2A shows the average number of cells per milliliter after a 24-h period of growth, and from these results we calculated the average doubling time (Fig. 2B) for each cell line. Compared to that of wild-type Y2 cells, the doubling time of RiD-2 cells was slightly increased (8.8 versus 8.3 h), whereas the doubling time of RiD-1 cells was 10.9 h. This corresponds to about a 30% increase in the duration of cell cycle and is similar to the growth rate defect of our previously published *ago1−/−* cells (21). Cells in which the *AGO1* deletion was complemented by reintroducing one copy of the *AGO1* gene at its chromosomal location (*ago1c*) had only a slight growth defect (8.5 versus 8.3 h) compared to wild-type cells.

In order to identify the potential cell cycle defect(s) underlying the increase in cell cycle length in RiD-1 and *ago1−/−* cells, we labeled DNA with propidium iodide and analyzed the DNA contents of the various cell populations by FACS analysis. As shown in Table 1, wild-type, RiD-1, RiD-2, *ago1−/−*, and *ago1c* trypanosomes had similar distributions of cells with DNA contents corresponding to 2C (cells in G₁), 4C (cells in G2 plus postmitotic cells), or between 2C and 4C (cells in S phase). However, both *ago1−/−* and RiD-1 cells showed approximately a 1.5- to 2-fold increase in the proportion of cells with a DNA content greater than 4C. In contrast, among the five cell lines we analyzed we could not detect any statistically

![FIG. 2. (A) Growth characteristics of wild-type (Y2), RNAi-deficient (RiD-1 and RiD-2), *ago1−/−*, and *ago1c* (ago1−/− cells complemented with one allele of *AGO1*) cells. Cells were counted and diluted to 5 × 10⁵/ml every 24 h for six consecutive cycles. The average number of cells after a 24-h period of growth is shown for each cell line. (B) The doubling time of each cell line was calculated from the data shown in panel A by using the equation $\log(2) = \log(x)/y$, where $x$ is the number of generations and $y$ is the number of cells times 10⁵/ml as determined 24 h after seeding; 24 is the doubling time. Error bars indicate standard deviations.](image)

| Cell type | % DNA content (mean ± SD) |
|-----------|-------------------------|
| <2C | 2C | >2C and <4C | 4C | >4C |
| Y2 | 1.1 ± 0.22 | 45.3 ± 2.76 | 19.9 ± 1.01 | 30.9 ± 2.93 | 3.3 ± 0.37 |
| *ago1−/−* | 1.6 ± 0.50 | 42.5 ± 1.28 | 19.7 ± 1.95 | 30.6 ± 2.18 | 6.3 ± 0.90 |
| *ago1c* | 0.4 ± 0.04 | 48.0 ± 2.47 | 20.3 ± 1.29 | 29.2 ± 2.49 | 2.5 ± 0.19 |
| RiD-1 | 1.2 ± 0.42 | 48.9 ± 3.56 | 18.9 ± 1.17 | 26.1 ± 3.02 | 5.3 ± 0.35 |
| RiD-2 | 1.0 ± 0.17 | 46.7 ± 4.30 | 20.2 ± 1.48 | 29.1 ± 3.34 | 3.5 ± 0.24 |
significant difference in the percentage of cells with a DNA content less than 2C. Cells in this category would include zoids (20), namely, cytoplasmic fragments containing kinetoplast (mitochondrial) DNA but devoid of nuclei.

Next, the cell cycle statuses of the various cell populations were monitored by using DAPI staining to visualize the nucleus and kinetoplast in each cell. The cell cycle position of trypanosomes can be readily defined by the nucleus and kinetoplast number in individual cells: G1 and S-phase cells have one kinetoplast and one nucleus (1K1N), G2 cells have two kinetoplasts and one nucleus (2K1N), postmitotic cells have two kinetoplasts and two nuclei (2K2N), and in some instances had completed a second round of mitosis (cells with more than two nuclei). Thus, stuck cells were derived from trypanosomes that had not completed cytokinesis at the previous cell cycle.

**RiD-1 and RiD-2 trypanosomes are defective in degradation of target mRNA but accumulate wild-type levels of siRNAs.** To define which step in the RNAi pathway was affected in RiD trypanosomes, we monitored the extent of degradation of target mRNA upon electroporation of wild-type (Y2), RiD-1, and RiD-2 cells with increasing doses of each of two different dsRNA triggers, namely, α-tubulin and PFR dsRNAs. Total RNA for Northern blot analysis was isolated 2 h after transfection, as mRNA degradation is maximal at 1 to 2 h after transfection with dsRNA (19). In addition, a sample of each cell culture was grown for 16 h to allow expression of the FAT phenotype. Although RNAi downregulation of PFR expression results in defects in the assembly of the paraflagellar rod in the new flagellum (1), we did not analyze this phenotype in this study. Figure 4 shows that, with 1, 2, or 5 µg of α-tubulin dsRNA, wild-type cells gave rise to 57, 81, or 92% FAT cells, respectively. In contrast, under the same conditions, RiD-1 and RiD-2 clones showed a dramatic impairment in their ability to respond to α-tubulin dsRNA challenge. In particular, at concentrations of dsRNA below saturation, namely, 1 and 2 µg/ transfection, both clones showed either undetectable amounts of FAT cells or about 0.5 or 1.0% FAT cells, respectively. However, at 5 µg of α-tubulin dsRNA/transfection, RiD-1 and RiD-2 clones gave rise to about 5 and 7% FAT cells, respectively. These results were consistent with the interpretation that RiD-1 and RiD-2 represented cell lines that have a severe impairment to challenge by α-tubulin dsRNA, but retain the

![FIG. 3. Morphology of RiD-1 cells defective in the last stages of cytokinesis. The gallery of images shows trypanosomes that had not completed cytokinesis and reinitiated a new cell cycle still being attached at the posterior end ("stuck" phenotype), with four kinetoplasts and two nuclei (A), four kinetoplasts and three nuclei (B), or four kinetoplasts and four nuclei (C). DNA was stained with DAPI, the flagellum was stained with an antibody recognizing the paraflagellar rod, and newly synthesized tubulin was stained with the Y1/2 monoclonal antibody. Arrowheads point to the kinetoplast DNA.](image)
ability to respond to high concentrations of the dsRNA trigger. Northern analysis confirmed that RiD-1 and RiD-2 trypanosomes are defective in the degradation of target α-tubulin or PFR mRNA (Fig. 4). In particular, RiD-2 cells showed a three- to fivefold decrease in the efficiency of target mRNA degradation. In contrast, the RNAi deficiency of RiD-1 cells was much more pronounced: at the concentrations of dsRNA tested, we could not detect significant degradation of target mRNA in RiD-1 cells. However, at higher dsRNA doses, namely, 10 or 20 μg of dsRNA, a limited amount of target mRNA degradation was also evident in RiD-1 cells (data not shown). From this analysis, we concluded that both RiD-1 and RiD-2 cells were impaired at the effector step of RNAi, namely, degradation of target mRNA.

The observed mRNA degradation defect in RiD-1 and RiD-2 cells could result from inefficient production or accumulation of siRNAs (the initiation step) or could represent a true block at the effector step of the RNAi pathway. To distinguish between these possibilities, we first monitored endogenous siRNAs homologous to the Ingi (Fig. 5A) or SLACS (Fig. 5B) retroposon transcripts by Northern blot hybridization with RNAs derived from Y1, Y2, RiD-1, and RiD-2 cells. This showed that the accumulation of Ingi or SLACS siRNA was not impaired in RiD-1 and RiD-2 cells, compared to their corresponding parent cell lines. Second, we examined whether RiD-1 and RiD-2 cells were defective in the accumulation of siRNAs derived from a transgene. To this end, we generated Y1, Y2, RiD-1, and RiD-2 cell populations constitutively expressing GFP dsRNA as a hairpin RNA (Y1-gfph, Y2-gfph, RiD-1-gfph, and RiD-2-gfph). All four cell lines tested expressed transcripts diagnostic of GFP dsRNA (data not shown). Northern analysis revealed that GFP siRNAs (Fig. 5C) were present in the RNAs derived from RiD-1-gfph (lane 2) and RiD-2-gfph (lane 4) cells in amounts similar to, if not higher than, those present in Y1-gfph (lane 1) and Y2-gfph (lane 3) RNAs. Since the GFP dsRNA-expressing cells represented populations rather than clones, it was possible that the RiD-1 and RiD-2 derivatives had a higher copy number of integrated GFP dsRNA transgenes and that this resulted in larger-than-wild-type amounts of GFP siRNAs. Taken together, the results presented in Fig. 4 and 5 demonstrated that RiD-1 and RiD-2 trypanosomes were wild type with respect to...
RiD-1 and RiD-2 trypanosomes accumulate increased amounts of retroposon-derived transcripts. Our previous analysis of AGO1 function in T. brucei revealed that genetic ablation of AGO1 resulted in upregulation of retroposon transcripts (21). Thus, we analyzed retroposon transcript abundance in RiD-1 and RiD-2 cells by Northern blotting. Figure 7A shows that, compared to their respective parent Y1 (lane 1) and Y2 (lane 3) cell lines, both RiD-1 (lane 2) and RiD-2 (lane 4) displayed a three- to fivefold increase in the abundance of the heterogenous transcripts homologous to the ubiquitous Ingi retroelement (18). Similar results were obtained for the SLACS transcripts (which are detected as a major band of about 6,000 nt [21]) in RiD-1 (Fig. 7B, compare lanes 1 and 6) or RiD-2 (data not shown) trypanosomes. This analysis showed that RiD cells had the hallmark of RNAi deficiency with respect to the accumulation of retroposon transcripts.

The increase in the steady-state level of retroposon transcripts could be a result of posttranscriptional and/or transcriptional mechanisms. To evaluate this, we chose RiD-1 cells, because they displayed the highest degree of RNAi deficiency. First, we analyzed the decay rate of SLACS transcripts after halting transcription with actinomycin D (Fig. 7B and C). In contrast, Ingi transcripts do not lend themselves to this analysis because they are too heterogeneous in size. The results showed that the half-life of SLACS transcripts in RiD-1 cells was approximately doubled compared to that in wild-type cells (Fig. 7C). Next, we quantitated the transcription of both SLACS and Ingi retroelements by hybridizing radiolabeled RNA synthesized in permeable cells to gene-specific fragments immobilized on a membrane (Fig. 7D). As controls for this experiment, we used RNAs derived from wild-type as well as from ago1Δ cells. Relative to that in the wild-type control, transcription of Ingi and SLACS retroelements was increased in RiD-1 cells, and the magnitude of the increase was similar to that observed in ago1Δ cells.

**DISCUSSION**

Here we have exploited the ability of α-tubulin dsRNA to block cytokinesis in wild-type cells to enrich for trypanosomes that were unable to respond to this challenge. We assumed that among the α-tubulin dsRNA-resistant cells, we might be able to identify trypanosomes defective in the RNAi pathway. The selection scheme was indeed successful and resulted in the isolation of RNAi-deficient cells. This in- vestigation, we used RNAs derived from wild-type as well as from ago1Δ cells. Relative to that in the wild-type control, transcription of Ingi and SLACS retroelements was increased in RiD-1 cells, and the magnitude of the increase was similar to that observed in ago1Δ cells.

We therefore applied the same selection procedure to two other trypanosome strains of independent origin, we found that seven to eight selection cycles were required to obtain relatively homogeneous populations resistant to challenge with α-tubulin dsRNA. This indicated that there are strain differences with respect to the recovery of RNAi-deficient cells by our selection procedure.
The higher rate of acquisition of RNAi deficiency in our laboratory strain might be due to heterozygosity at one or more loci that carry genes that are directly or indirectly involved in the RNAi pathway.

The RiD-1 and RiD-2 clones were chosen for detailed analysis, because they were derived from independent selections with α-tubulin dsRNA and because they showed the highest degree of resistance to challenge with α-tubulin dsRNA, as initially measured by their deficiency in acquiring the FAT phenotype. Nevertheless, analysis of 30 clonal lines, derived from α-tubulin dsRNA-resistant Y1 and Y2 cell populations, revealed heterogeneity among the clones in the degree of resistance to α-tubulin dsRNA. Cumulatively, about 25% of the clones displayed between 1 and 2% FAT cells, about 50% gave rise to between 5 and 10% FAT cells, and the remaining clones displayed more than 10% FAT cells (data not shown). These observations are consistent with the possibility that acquisition of resistance to α-tubulin dsRNA occurred independently in several cells of the Y1 or Y2 starting cell population. However, since we did not identify the genes responsible for the RiD-1 or RiD-2 phenotype, it is unclear whether RiD-1 and RiD-2 might be allelic.

In principle, identification of the genetic lesion(s) in RiD-1 and RiD-2 trypanosomes could be achieved by genetic complementation. Genetic complementation was reported several years ago for one mutant *T. brucei* strain, namely, a null mutant for the ornithine decarboxylase gene (22). To attempt complementation of RiD clones, we generated RiD-1 and RiD-2 reporter strains expressing GFP and GFP dsRNA. We reasoned that upon transfection with a genomic library, we might be able to select for cells that had lost GFP fluorescence, because the function of the RNAi pathway had been restored. However, after a few weeks in culture the reporter strains accumulated a considerable number of cells that no longer expressed GFP. This indicated that in some cells the GFP gene had been “naturally” silenced, thus negating the possibility for complementation.
At the cellular level, RiD-1 and RiD-2 cells displayed different growth characteristics. Both the doubling time and the cell cycle status of RiD-2 cells were similar, but not identical, to those of wild-type cells. In contrast, RiD-1 cells showed approximately a 30% delay in cell division, as well as accumulation of “stuck” cells that were unable to terminate cytokinesis. Intriguingly, these abnormalities closely paralleled those of the ago1−/− cells that we previously established in our laboratory strain. However, the small proportion of stuck cells that we detected in RiD-1 and ago1−/− trypanosomes does not seem sufficient to account for the observed 30% increase in the duration of the cell cycle. It is likely that the number of cells with defects at terminating cytokinesis might have been underestimated in our analysis, because the stuck phenotype could be identified with certainty only if cells had reentered the cell cycle. Defects in the last stages of cytokinesis in S. pombe mutants lacking the dicer1 or ago1 gene have been recently reported (2). In this case it was shown that ago1 or dicer1 is required for regulated hyperphosphorylation of Cdc2 kinase when encountering genotoxic insults and that this results in abnormal cytokinesis, cell cycle arrest deficiencies, and mating defects. In contrast, in T. brucei there seems to be no nuclear mitosis/cytokinesis checkpoint in that cytokinesis can proceed in the absence of mitosis (20) and, conversely, kinetoplast segregation and mitosis can occur in the absence of cytokinesis (19). These observations point to fundamental differences between trypanosomes and other eukaryotes in terms of cell cycle control. Clearly, understanding of how RNAi deficiency affects the cell cycle of T. brucei requires further investigation. Lastly, it should be noted that the growth defects we observe in the RNAi-deficient cells established in our trypanosome laboratory strain are far less dramatic than those reported for an ago1 null cell line derived from T. brucei 427 procycls (7). One possibility to reconcile these different results is that the severity of the cellular consequences which are observed upon eliminating or downregulating the RNAi pathway is influenced by the genetic backgrounds of the different trypanosome strains used for the experiments. It is also possible, as we discussed previously (25), that the cellular defects observed upon elimination of the RNAi pathway may result at least in part, from the accumulation of mutations due to the potential mobilization of retroelements in RNAi-deficient cells. We have recently constructed a strain in which the ago1 gene is conditionally expressed. Thus, it should now be possible to assess the timing of appearance and extent of cellular defects when RNAi is downregulated and to distinguish between direct and indirect effects of RNAi deficiency.

Both RiD-1 and RiD-2 trypanosomes turned out to be deficient in the RNAi pathway, although the RNAi deficiency was not absolute, as these cells retain a limited capacity to carry out degradation of target mRNAs at high concentrations of trigger dsRNA. One possible explanation of this phenomenon is that at high concentrations of trigger dsRNA there should be a greater proportion of AGO1 loaded with the specific siRNAs, and this might bypass to some extent the defect(s) in RiD cells. The observation that both RiD clones were severely impaired in degradation of two different target mRNAs indicated that the RNAi deficiency was not restricted to α-tubulin mRNA. Since accumulation of endogenous retroposon-derived or transgene-derived siRNAs was not affected, we concluded that the effector step of RNAi, namely, degradation of target mRNA, was impaired. However, the RNAi-deficient phenotype of RiD-1 and RiD-2 cells was not a consequence of mutations in the ago1 gene or of defects in the expression level or cellular distribution of the AGO1 protein. Taken together, our results indicated that neither the processing of dsRNA nor AGO1 was altered in RiD trypanosomes. Rather, the phenotypes of RiD cells point to a step affecting the efficiency of cleavage of mRNA by AGO1. It is possible that the RiD genetic lesion(s) affects the function of one or more components required to interact with AGO1 to bring about efficient cleavage of mRNA. In this scenario, in vivo the endonuclease activity of AGO1 might be regulated by interaction with this putative factor(s). A second possibility is that the activity of AGO1 might be regulated by posttranslational modifications, such as phosphorylation or methylation, which are also known to modulate protein-protein interactions. We have recently found that AGO1 is posttranslationally methylated at arginine residues in the amino-terminal RGG domain (N. Chamond et al., unpublished observation), thus supporting the hypothesis that AGO1 function might be modulated by methylation. Once the function of these modified arginine residues is understood, it will be interesting to determine whether AGO1 in RiD cells is appropriately modified.

Both RiD clones had a pronounced increase in the steady-state level of retroposon transcripts, the hallmark of genetic ablation of the RNAi pathway in trypanosomes. Similar to the case for ago1−/− cells (21), the increased steady-state level of SLACS retroposon transcripts in RiD-1 cells was brought about by an increase in the stability of the retroposon transcripts and in the transcription of the corresponding retroelements. However, it should be pointed out that the increase in stability of SLACS transcripts in RiD-1 cells was not as pronounced as that in ago1−/− cells, namely, a twofold increase in RiD-1 cells versus a fourfold increase in ago1−/− cells (21). This difference could be explained by the fact that RiD-1 cells still retain some ability to cleave mRNA, whereas ago1−/− cells are functionally RNAi negative.

The observation that RiD-1 trypanosomes display an increased transcription of retroelements, which is similar to that of ago1−/− cells, further supports the possibility that the RNAi pathway has a role, either direct or indirect, in regulating transcription of retroelements. By analogy to the function of the RNAi pathway in heterochromatin formation in S. pombe (27), it is plausible that in T. brucei the absence of a functional RNAi pathway results in changes in chromatin structure leading to an increased accessibility of retroelements to the RNA polymerase II transcriptional machinery. On the other hand, one could imagine that the retroposon transcripts themselves or perhaps the proteins encoded by retroposon mRNAs might have a role in regulating transcription of the elements in the genome.

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