Interactions between the RNA Interference Effector Protein Ago1 and 14-3-3 Proteins

CONSEQUENCES FOR CELL CYCLE PROGRESSION*

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The Argonaute family member Ago1 is required for formation of pericentric heterochromatin and small interfering RNA (siRNA)-mediated post-transcriptional gene silencing in the fission yeast Schizosaccharomyces pombe. In addition, we have recently demonstrated that Ago1 function is required for enactment of cell cycle checkpoints (Carmichael, J. B., Provost, P., Ekwall, K., and Hobman, T. C. (2004) Mol. Biol. Cell 15, 1425–1435). Here, we provide evidence that the amino terminus of Ago1 binds to proteins that function in cell cycle regulation including 14-3-3 proteins. Interestingly, the amino terminus of human Ago2, the endonuclease that cleaves siRNA-targeted mRNAs, was also demonstrated to bind 14-3-3 proteins. Overexpression of the Ago1 amino terminus in yeast resulted in cell cycle delay at the G2/M boundary. Further investigation revealed that nuclear import of the mitosis-inducing phosphatase Cdc25 is inhibited by overexpression of the Ago1 amino terminus. Under these conditions, we found that the cyclin-dependent kinase Cdc2 is constitutively phosphorylated on tyrosine 15, thereby reducing the activity of this kinase, a situation that delays entry into mitosis. We hypothesize that 14-3-3 proteins are required for Argonaute protein functions in cell cycle and/or gene-silencing pathways.

Argonaute proteins function in RNA interference (RNAi) (4), a gene-silencing process that occurs in most eukaryotes with the exception of budding yeast. Members of the Argonaute family form the core of ribonucleoprotein complexes that effect gene silencing by targeted mRNA cleavage, translational repression, or chromatin modification (reviewed in Refs. 5 and 6). Small RNAs that are bound to the Argonaute subunit target the ribonucleoprotein complexes to homologous mRNAs or genomic loci. It has been estimated that Argonaute-dependent RNAi controls the expression of more than 30% of genes in the human genome (7). Many of the genes regulated by the RNAi pathway encode proteins including transcription factors that regulate signaling pathways and cell cycle progression.

The study of RNAi in the fission yeast Schizosaccharomyces pombe has led to a number of important breakthroughs including the realization that, in addition to functioning at the post-transcriptional level (2), this pathway regulates chromatin structure and transcriptional gene silencing (1, 8). We are interested in the relationship between the yeast RNAi apparatus and cell cycle progression. Unlike in higher eukaryotes, the central RNAi effector proteins in yeast, Ago1, Dcr1, and Rdp1, do not appear to play a major role in regulating global gene expression (9, 10). Transcriptome profiling revealed that the yeast RNAi apparatus regulates the expression of only 42 transcripts. Of the genes that are negatively regulated by RNAi, none have defined roles in cell proliferation pathways. Rather, many of these transcripts are noncoding and originate from centromeric repeats and retrotransposons. Our recent work suggests that yeast Argonaute and Dicer proteins function in cell cycle progression in a pathway that does not require small interfering RNAs (3). In addition, human Ago2, a central component of the RNA-induced silencing complex (11), was shown to complement the checkpoint defects in ago1 null mutants (3), suggesting that Argonautes have the capacity to regulate cell cycle progression in higher eukaryotes. To further investigate the role of Argonautes in cell cycle events, we employed a dominant negative strategy to disrupt cell cycle progression in yeast. In this study, we report that Argonaute proteins interact with 14-3-3 proteins. In yeast, these interactions are important for regulating the phosphorylation state of the cyclin-dependent kinase, Cdc2.

EXPERIMENTAL PROCEDURES

Materials—and list of S. pombe strains used in this study and their corresponding genotypes are shown in Table 1. Unless otherwise indicated, yeast were cultured at 30 °C in yeast extract or essential minimal medium (EMM) lacking nutritional supplements as described (12). The transformation of yeast strains was performed using the alkaline cation method (12). To induce expression of genes under the control of the nmt (no message thiamine) promoter, cultures were grown overnight in EMM lacking thiamine. The Escherichia coli strain DH5α was used for propagation of plasmids (Table 2).

Plasmid Construction—for expression in S. pombe, a cDNA encoding Ago1 was amplified by PCR using primers AgoSall-F...
5'-GGGGTCAGCATGTCGATAT-3' and Ago1SalI-R 5'-GTGGGTATATGTGAAGTCGACCC-3' with Expand High Fidelity polymerase (Roche Applied Science) and then subcloned into the shuttle vector pGEM-T (Promega). The ago1 fragment was excised with Sall and then ligated in frame to the 3' end of the GST cassette of pDS473a (13) to create GST-Ago1. The GST-NT-Ago1 and GST-CT-Ago1 expression plasmids were constructed by excising the 5' and 3' halves of the ago1 cDNA from pGEM-T-Ago1 using Sall and BglII or BglII and SmaI, respectively, followed by ligation into the pDS473a vector. Red fluorescent protein (RFP)-tagged NT-Ago1 was constructed as follows. The RFP cassette and NT-Ago1 fragment were amplified by PCR using the primer sets nRFPfNotI-F (5'-ATATGAGCTCTTAAGATCTAATAGGAG-3') and Ago1SalI-R (5'-ATATGGTACCTCCTCCTTGTAC-3'), RFP-4Gly-KpnI-R (5'-ATATGGTACCTCCTCCTTGTAC-3'), RFP-NotI-F (5'-ATATGAGCTCTTAAGATCTAATAGGAG-3'), RFP-NotI-R (5'-ATATGGTACCTCCTCCTTGTAC-3') and Ago1SalI-R (5'-ATATGGTACCTCCTCCTTGTAC-3'). The resulting RFP and NT-Ago1 products were digested with NotI and KpnI and with NotI and KpnI, respectively. The fragments were subcloned via a three-part ligation reaction into the NotI and SacI site of the pAALN vector (14). For some experiments, we used Ago1 expression plasmids that contained a G418 resistance marker pAALN vector (14). For some experiments, we used Ago1 products were digested with NotI and KpnI and with NotI and KpnI, respectively. The resulting plasmids were subjected to mass spectrometry at the Institute for Biomolecular Design (University of Alberta).

Where indicated, GST-Argonaute fusion proteins were transiently expressed in HeLa cells, and binding proteins were isolated as described (15). The association of 14-3-3 proteins with GST-Ago1 was confirmed by using rabbit polyclonal sera to 14-3-3 proteins (Santa Cruz Biotechnology). Hydroxyurea (HU) Survival Assay—Serial dilutions of actively growing yeast cultures were spotted onto EMM media lacking uracil in the presence or absence of the S-phase arrest-inducing drug, HU (3.5–10 mM). After 3–5 days at 30 °C, samples were scored for growth.

Septation Analyses—Yeast strain FY254 was transformed with pDS473a, pDS473a-Ago1, or pDS473a-NT-Ago1 and then cultured at 30 °C in EMM lacking uracil to mid-log phase (A695 = 0.6 – 0.9), treated with 10 mM HU for 4 h, washed once in EMM lacking uracil, resuspended in EMM lacking uracil, and incubated for an additional 4 h. The numbers of septated cells in each sample were determined at hourly intervals pre- and post-treatment with hydroxyurea. For each sample, at least 300 cells were scored.

GST Pulldowns—Bacteria transformed with pGEX4T-1 or pGEX4T-1-NT-Ago1 were grown to an A660 of 0.2. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM to induce expression of the fusion proteins, and the cultures were incubated for a further 4 h at 37 °C. Bacteria were harvested by centrifugation at 3000 × g for 10 min at 4 °C and then resuspended in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and Complete™ EDTA-free protease inhibitors (Roche Applied Science). Cells were lysed by sonication, and the resulting lysates were clarified by centrifugation at 1,000 × g for 5 min at 4 °C. Lysates were prepared from S. pombe strains as follows. Cell pellets (500 µl) were resuspended in 1 ml of PBS + 0.1% Triton X-100 containing Complete™ EDTA-free protease inhibitors (Roche Applied Science). Acid-washed glass beads (Sigma) were added to samples (50% volume), which were then subjected to four cycles of homogenization at 4 °C by bead beating (Mini Beadbeater-8, BioSpec Products). The resulting slurries were clarified by centrifugation (1000 × g) for 10 min.

Glutathione-Sepharose beads (Amersham Biosciences), washed three times in PBS + 0.1% Triton X-100 and incubated by rotation with bacterial lysates containing the expressed fusion protein for 3 h at 4 °C. Fusion protein-glutathione bead complexes were washed three times in PBS + 0.1% Triton X-100 containing protease inhibitors and incubated with the yeast lysates overnight at 4 °C on a rotator. Bound protein complexes were washed three times with PBS + 0.1% Triton X-100 containing protease inhibitors, resuspended in 5× sample buffer, and boiled. Protein complexes were resolved by SDS-PAGE and detected by Coomassie Blue staining or immunoblot analysis. Proteins excised from Coomassie Blue-stained gels were subjected to mass spectrometry at the Institute for Biomolecular Design (University of Alberta).

Where indicated, GST-Argonaute fusion proteins were transiently expressed in HeLa cells, and binding proteins were isolated as described (15). The association of 14-3-3 proteins with human Ago2 fusion proteins was detected by immunoblotting using rabbit polyclonal sera to 14-3-3 proteins (Santa Cruz Biotechnology).

Cdc2 Phosphorylation Assay—FY254 expressing GST or GST-NT-Ago1 was cultured at 30 °C in EMM lacking uracil to A695 = 0.3, after which time the samples were divided into two aliquots. HU was added to one of the aliquots to a final concentration of 10 mM. Incubation of the cultures was continued for an additional 3 h. Lysates were prepared by mechanically breaking cells in radioimmune precipitation assay buffer with glass beads using a Beadbeater-8 device. Cells were subjected to
Ago1 Interacts with Cell Cycle Machinery

RESULTS

Overexpression of the Ago1 N Terminus Affects Cell Cycle Progression—We had previously demonstrated that Ago1 function is required to enact the S-phase replication checkpoint in fission yeast (3). As a consequence, ago1 null mutants are unable to survive in the presence of HU. Our strategy was to identify a region of Ago1 that when overexpressed would confer sensitivity to HU, presumably by preventing enactment of the replication checkpoint. Regions of the Ago1 cDNA that included the PAZ (NT-Ago1) or PIWI (CT-Ago1) domain-encoding regions were subcloned downstream from the GST coding regions were subcloned downstream from the GST cassette in the expression vector pDS473a (Fig. 1A). The plasmids were transformed into the S. pombe haploid strain FY254. Yeast were cultured in the absence of thiamine to induce overexpression of the GST-Ago1 fusion proteins. Cultures were spotted onto EMM with or without HU and scored for growth. Overexpression of the amino-terminal region of Ago1 (NT-Ago1) caused marked hypersensitivity to HU (Fig. 1B). Relative to yeast overproducing GST alone, the CT-Ago1 strain exhibited a slight growth defect on HU but otherwise appeared normal when examined by light microscopy. Microscopic examination of yeast cells overexpressing the NT-Ago1 protein revealed that they were relatively large and contained prominent but abnormal nuclei (Fig. 1C). The DAPI-stained nuclei of NT-Ago1 overproducers were noticeably larger than the other strains, an observation that prompted us to use FACS analyses to determine whether these cells contained more nuclear DNA than cells overexpressing GST or CT-Ago1. Because fission yeast have an unusually long G2-phase (16) and also begin replication prior to completion of cytokinesis, the bulk of exponentially growing cells in a haploid strain exhibit a 2N DNA profile. As expected, the majority of yeast overexpressing GST or CT-Ago1 exhibited a 2N content of DNA (Fig. 1C). In contrast, the DNA content of NT-Ago1-expressing cells was predominantly 4N, suggesting that these cells had rereplicated their DNA and were delayed at the G2/M boundary.

To further examine the nature of the NT-Ago1-dependent HU hypersensitivity, we monitored the cell cycle progression of this strain during and after exposure to HU. Normally, when exposed to replication stress, yeast will activate the replication checkpoint to arrest the cell cycle until the stress is relieved. Checkpoint activation in yeast can be monitored microscopically by scoring for the presence of septa, a cellular structure that functions in cell division (17). Because the onset of mitosis precedes septum formation in fission yeast, blocking the cell cycle prior to the onset of mitosis leads to a decrease in the number of septated cells. Thus, in an actively growing population of yeast cells, replication stress will result in cell cycle delay.

Microscopy—Strains were grown to mid-log phase, collected by centrifugation (1000 × g) for 5 min, resuspended in a DAPI/water solution (1 μg/ml), and placed on slides coated with agarose (0.8%). Yeast transformed with plasmids containing a G418 resistance marker was cultured in medium containing 200 μg/ml G418. Where indicated, cultures were treated with leptomycin B (100 ng/ml) for 1–2 h prior to analyses. Samples were viewed using an Ultraview ERS spinning disc confocal imager (PerkinElmer Life Sciences) attached to an Axiovert 200 M microscope (Zeiss).

Flow Cytometry—Aliquots of 10^6 cells were collected from mid-log liquid cultures, pelleted, and fixed by resuspension in 1 ml of 70% ethanol. Cells were washed in 3 ml of 50 mM sodium citrate, resuspended in 0.5 ml of 50 mM sodium citrate containing 0.1 mg/ml RNase A, and incubated at 37 °C for 2 h. Cells were stained by adding 0.5 ml of sodium citrate solution containing 2 mM Sytox Green (Molecular Probes) and stored at 4 °C until processed. Cells were vortexed for 45 s prior to analyses on a BD Biosciences FACScan. For each sample, 20,000 cells were analyzed.
and concomitant decreased septation. As expected, yeast overexpressing GST alone exhibited a sharp decrease in their septation indices following exposure to HU (Fig. 2). Shortly after removal of HU, cell cycle progression resumed as evidenced by an increase in the proportion of septated cells. In contrast, yeast overexpressing GST-NT-Ago1 did not immediately delay their cell cycle but, instead, appeared to progress toward mitosis (Fig. 2). After this point, the cells were not able to recover from the HU treatment. It is likely that when exposed to HU, yeast expressing NT-Ago1 are unable to activate the S-phase checkpoint and die from “mitotic catastrophe” (18) as a result of entering mitosis with incompletely replicated chromosomes. From these results, we conclude that the NT-Ago1 construct acts as a dominant negative protein that competes for Ago1-specific interactions with the cell cycle apparatus.

Argonaute Proteins Bind to 14-3-3 Proteins—To gain insight into how NT-Ago1 may be exerting its effects on the cell cycle machinery, we used affinity purification to identify proteins that bind to the amino terminus of Ago1. The NT-Ago1 fusion was expressed in bacteria, isolated on glutathione-agarose beads (Fig. 3A), and incubated with lysates prepared from an ago1 null strain. Bound proteins were eluted from the agarose beads, separated by SDS-PAGE, and then visualized by Coomassie Blue staining (Fig. 3A). Although many of the proteins that eluted from the NT-Ago1 column were bacterial in origin, by using mass spectrometry we were able to identify a limited number of yeast proteins that bound to GST-NT-Ago1 but not GST (Fig. 3A). One of these proteins was Rad25, a member of the 14-3-3 family. The identification of Rad25 as an Ago1-interacting protein was of particular interest because 14-3-3 proteins have well documented roles in cell cycle transition and checkpoint enactment (19). Importantly, we were able to demonstrate an interaction between Rad25 and full-length endogenous Ago1 by reciprocal GST pulldown and immunoblotting assays (Fig. 3B). A Δago1 strain was used as a negative control for these experiments. Rad25 is closely related to a second S. pombe 14-3-3 protein, Rad24, that has similar functions (20). We also detected interactions between Rad24 and Ago1 (Fig. 3B); however, it is unclear whether Ago1 interacts directly with Rad24 or with Rad24/25 heterodimers (21). To determine whether binding interactions between 14-3-3 proteins and Argonautes have been conserved throughout evolution, we looked for interactions between 14-3-3 proteins and the RNAi slicer activity-containing protein, human Ago2 (22). Indeed, stable interaction between the PAZ-containing region of human Ago2, and 14-3-3 proteins was evident (Fig. 3C).

Overexpression of NT-Ago1 Affects Cdc25 Transport and Phosphorylation of Cdc2—Rad25 functions together with Rad24 to regulate cell cycle progression and enactment of checkpoints. For example, binding between Rad24/25 and Chk1, a protein kinase required for enacting the DNA damage checkpoint (23, 24), is stimulated by DNA damage (25). In response to DNA damage, one of the functions of activated Chk1 is to phosphorylate the mitotic inducer Cdc25, thereby creating 14-3-3 binding sites. For yeast and vertebrate isoforms of Cdc25, binding of 14-3-3 proteins induces export of this phosphatase from the nucleus (26, 27). Nuclear exclusion of Cdc25 correlates with an inability to enter mitosis because the phosphatase no longer has access to its substrate, the Cdc13-Cdc2 complex, which is located in the nucleus (27). Because dephosphorylation of Cdc2 by Cdc25 is required for transition from G2 to M phase (reviewed in Ref. 28), mutations that reduce
Cdc25 activity can result in elongated cells that are blocked at G₂/M (29).

Previously, we showed that Ago1 is required for phosphorylation of Cdc2 on tyrosine 15 in response to genotoxic stress (3). Accordingly, we reasoned that the elongated phenotype of NT-Ago1-expressing cells may result from an inability of Cdc25 to gain access to and dephosphorylate Cdc2, possibly via a mechanism that involves impaired nuclear import of Cdc25.

The yeast strain Q2017, which contains a GFP cassette genomically integrated at the \( cdc25 \) locus (Q2017). Samples were treated with (+) or without (−) leptomycin B (LMB). The localizations of Cdc25-GFP were examined by confocal microscopy. Bar = 2 μm. The localizations of Cdc25-GFP (green) are shown in yeast cells expressing RFP-NT-Ago1 or RFP alone. In the latter case, Cdc25-GFP is concentrated in the nuclei of a dividing cell. C, lysates from yeast expressing GST or GST-NT-Ago1 were subjected to SDS-PAGE and immunoblotting with antibodies to Cdc2 and phospho-Cdc2.

**FIGURE 4.** Overexpression of NT-Ago1 results in constitutive phosphorylation of tyrosine 15 on Cdc2. A, RFP alone or RFP-NT-Ago1 was overexpressed in a yeast strain that contains a GFP cassette genomically integrated at the \( cdc25 \) locus (Q2017). Samples were treated with (+) or without (−) leptomycin B (LMB). The localizations of Cdc25-GFP were examined by confocal microscopy. Bar = 2 μm. B, the localizations of Cdc25-GFP (green) are shown in yeast cells expressing RFP-NT-Ago1 or RFP alone. In the latter case, Cdc25-GFP is concentrated in the nuclei of a dividing cell. C, lysates from yeast expressing GST or GST-NT-Ago1 were subjected to SDS-PAGE and immunoblotting with antibodies to Cdc2 and phospho-Cdc2.

DISCUSSION

In the present study, we report for the first time that the Argonaute proteins bind to 14-3-3 proteins in yeast and human cells. Overexpression of the 14-3-3 binding region of Ago1 results in cell cycle delay at the G₂/M transition phase. We hypothesize that the dominant negative effects of NT-Ago1 are related to its ability to bind 14-3-3 proteins. The nature of Argonaute/14-3-3 interactions remained to be determined. Analyses of the Ago1 and human Ago2 sequence at high stringency using the Scansite algorithm revealed that, unlike Cdc25, no phosphorylation-dependent 14-3-3 binding motifs were evident. However, running the analyses at medium stringency revealed the presence of two predicted 14-3-3 binding sites in the amino-terminal region of human Ago2 (but not yeast Ago1). As such, it is not clear whether 14-3-3 bind directly to Argonaute proteins or as part of a complex.

Binding of 14-3-3 proteins to Cdc25 is required for checkpoint enactment in response to unreplicated DNA (32), and one theory purports that this interaction contributes to the nuclear exclusion of Cdc25 (27). Retention of Cdc25 in the cytoplasm is thought to prevent entry into mitosis. Conversely, after the replication stress is relieved, dissociation of the 14-3-3/Cdc25...
complex is required before mitosis can resume. In this scenario, it is possible that the NT-Ago1 protein stabilizes the 14-3-3-Cdc25 complex in the cytoplasm independently of the Cdc25 phosphorylation status. Indeed, this is how the Vpr protein of human immunodeficiency virus is thought to delay cell cycle progression at the G2/M boundary in mammalian cells by promoting association of 14-3-3 and Cdc25C in a manner that does not require phosphorylation of Cdc25 (35). However, given that we did not observe colocalization between Cdc25-GFP and RFP-NT-Ago1 in the cytoplasm, it seems unlikely that NT-Ago1 functions in a similar manner to Vpr.

We favor the idea that NT-Ago1 exerts a dominant negative effect by working upstream of Cdc25 interaction with 14-3-3 proteins. In this respect, it is interesting to note that 14-3-3 proteins have also been proposed to function in nuclear import of Cdc25 (32). Under this scenario, overexpression of NT-Ago1 may be expected to reduce the pool of available 14-3-3 proteins for cell cycle regulators such as Cdc25 by sequestering them into nonproductive complexes. This situation would also result in delayed mitosis because of constitutively high levels of phospho-Cdc2 in the nucleus. Finally, in light of previous work by Nurse and colleagues, which revealed that artificially lowering the activity of Cdc2 in G2 can lead to re-replication (37), it is likely that the 4N DNA content phenotype of NT-Ago1 overexpressing cells is caused by the constitutive inhibitory phosphorylation of Cdc2.

In summary, these data build upon previous work from our laboratory showing that ago1 is required for regulated phosphorylation of Cdc2 (3). The results are consistent with a scenario in which physical interactions between Ago1 and the cell cycle modulators Rad25 and Rad24 are required for this process. In turn, nuclear import of the mitotic inducer Cdc25 appears to be negatively affected. It will now be of interest to determine whether and how 14-3-3-dependent upstream regulators of Cdc25, such as Chk1, interact with Ago1 and potentially with its binding partner, Dcr1. In this respect, it is interesting to note that both Chk1 and human Ago2 are Hsp90 substrates and that it is the amino-terminal region of Ago2 that binds to Hsp90 (15, 36). Finally, it is also important to consider the possibility that 14-3-3 proteins are required for the function of Argonaute proteins in gene-silencing pathways.

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