Hsp90 Regulates the Function of Argonaute 2 and Its Recruitment to Stress Granules and P-Bodies

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Submitted January 28, 2009; Revised May 4, 2009; Accepted May 11, 2009

Monitoring Editor: Marvin Wickens

Argonaute proteins are effectors of RNA interference that function in the context of cytoplasmic ribonucleoprotein complexes to regulate gene expression. Processing bodies (PBs) and stress granules (SGs) are the two main types of ribonucleoprotein complexes with which Argonautes are associated. Targeting of Argonautes to these structures seems to be regulated by different factors. In the present study, we show that heat-shock protein (Hsp) 90 activity is required for efficient targeting of hAgo2 to PBs and SGs. Furthermore, pharmacological inhibition of Hsp90 was associated with reduced microRNA- and short interfering RNA-dependent gene silencing. Neither Dicer nor its cofactor TAR RNA binding protein (TRBP) associates with PBs or SGs, but interestingly, protein activator of the double-stranded RNA-activated protein kinase (PACT), another Dicer cofactor, is recruited to SGs. Formation of PBs and recruitment of hAgo2 to SGs were not dependent upon PACT (or TRBP) expression. Together, our data suggest that Hsp90 is a critical modulator of Argonaute function. Moreover, we propose that Ago2 and PACT form a complex that functions at the level of SGs.

INTRODUCTION

The Argonaute superfamily comprises a group of RNA-binding proteins that form the cores of ribonucleoprotein complexes (RNPs) that mediate RNA interference (RNAi) and related gene-silencing pathways (reviewed in Hannon, 2002; Peters and Meister, 2007; Hutvagner and Simard, 2008). All metazoans encode multiple Argonaute genes, the products of which have unique as well as overlapping functions. The basic functions of Argonautes in the canonical RNAi pathway are reasonably well understood. Small guide RNAs direct Argonaute-containing RNPs to homologous mRNAs, thereby providing specificity in this pathway (reviewed in Hannon, 2002; Jaronczyk *et al.*, 2005). The Argonaute superfamily has been divided into two subgroups, Argonaute and Piwi (Bohmer *et al.*, 1998; Cox *et al.*, 1998; Carmell *et al.*, 2002). Expression of the Piwi subfamily members is restricted to germline tissues and undifferentiated cells, whereas the Argonaute group members are ubiquitous. The two main types of small RNAs that associate with Argonaute subfamily members differ in origin and function. Short interfering RNAs (siRNAs) and microRNAs (miRNAs) are derived from long double-stranded (ds)RNA and hairpin RNA precursors, respectively. Successive processing of miRNA precursors in the nucleus and cytoplasm, respectively, by the RNases Drosha (Lee *et al.*, 2003) and Dicer (Bernstein *et al.*, 2001) produces mature miRNAs that are then transferred to an Argonaute-containing RNP complex

termed RNA induced silencing complex (RISC) (Hammond *et al.*, 2000). The biogenesis of siRNAs only requires Dicer activity. A third species of small noncoding RNAs (Piwi-interacting RNAs) that associate with Piwi subfamily members was recently discovered (Aravin *et al.*, 2006; Girard *et al.*, 2006). However, their expression is limited to germline tissues in which they are thought to function in gametogenesis and retrotransposon silencing (reviewed in Klattenhoff and Theurkauf, 2008).

It is estimated that expression of 30% of all human genes may be controlled posttranscriptionally by miRNA-dependent mechanisms (Lewis *et al.*, 2005). In addition, the RNAi apparatus acts in the nucleus to regulate gene expression at the transcriptional level (Janowski *et al.*, 2006; Kim *et al.*, 2006; Tam *et al.*, 2008). Because of its central role in gene expression, the RNAi apparatus itself is subject to regulation, and evidence suggests that this occurs at multiple levels. For example, processing of miRNA precursors in the nucleus by Drosha varies in a developmental stage- and cell-specific manner (Thomson *et al.*, 2006). After processing, the export of pre-miRNA intermediates is controlled by the karyopherin exportin 5 (Bohnsack *et al.*, 2004). Regulation of si- and miRNA pathways also occurs at multiple points in the cytoplasm. Loading of small RNAs onto mammalian Argonaute complexes seems to be facilitated by a complex of RNA-binding proteins that includes Dicer, TAR RNA binding protein (TRBP), and protein activator of the double-stranded RNA-activated protein kinase (PACT) (Maniataki and Mourelatos, 2005; Lee *et al.*, 2006). Finally, localization and presumably the functions of Argonaute proteins are regulated by phosphorylation (Zeng *et al.*, 2008).

In the cytoplasm, Argonaute-dependent posttranscriptional gene silencing (PTGS) has been linked to discrete cytoplasmic puncta called GW- or P-bodies (Jakymiw *et al.*, 2005; Liu *et al.*, 2005; Sen and Blau, 2005). These structures

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E09-01-0082>) on May 20, 2009.

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are known to play important roles in mRNA catabolism and contain RNA decapping enzymes and exonucleases as well as Argonaute proteins. An ongoing matter of debate is whether these structures are required for small RNA-mediated PTGS or whether they simply form as a consequence of silencing. Regardless of which school of thought is ultimately proven correct, it is likely that formation of PBs may increase the efficiency or kinetics of PTGS. In addition to PBs, Argonautes have been shown to rapidly associate with stress granules (SGs) when cells encounter translational stress. Microscopically visible SGs are not present in “unstressed” cells; however, treatment with arsenite or the translational repressor hippuristanol results in rapid formation of SGs (Bordeleau *et al.*, 2006; Leung *et al.*, 2006). SGs contain stalled translation complexes and have been implicated in miRNA-mediated translational repression (Anderson and Kedersha, 2008). Given that targeting of Argonautes to PBs and SGs seem to be dynamic, modulating the association of Argonautes with these structures is a potential mechanism to modulate PTGS. Indeed, this process seems to be regulated in mammalian cells in that biogenesis of PBs is linked to maturation of miRNAs (Pauley *et al.*, 2006). In the present study, we provide evidence that localization and function of Ago2 and potentially other Argonaute proteins is regulated by the activity of a heat-shock protein (Hsp) 90 complex.

MATERIALS AND METHODS

Reagents

Stock solutions (1 mM) of hippuristanol (a gift from Dr. J. Pelletier, McGill University, Montreal, QC, Canada) and geldanamycin (Geld) (LC Laboratories, Woburn, MA) were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) and stored at -80°C . All reagents for mammalian cell culture were obtained from Invitrogen (Carlsbad, CA) or Sigma-Aldrich.

Antibodies

The rabbit polyclonal anti-Aha1 has been described previously (Wang *et al.*, 2006). Mouse monoclonal to human anti-Ago2 was a gift from Dr. Z. Mourelatos (University of Pennsylvania School of Medicine, Philadelphia, PA). A rabbit polyclonal antibody (2D4) was generated against the PAZ domain of hAgo2 in this laboratory. Human anti-GW was a gift from Dr. M. Fritzler (University of Calgary, Calgary, AB, Canada). Goat and rabbit anti-green fluorescent protein (GFP) and rabbit anti-cytochrome *c* antibodies were gifts from Dr. L. Berthiaume (University of Alberta, Edmonton, AB, Canada). Rabbit polyclonal antibodies to TRBP and PACT were gifts from Drs. A. Gatignol (McGill University) and G. Sen (Cleveland Clinic, Cleveland, OH), respectively. Commercially available antibodies were purchased from the following sources: mouse monoclonals anti-Hsp90 (SPA-830), anti-HOP (SRA-1500), anti-FKBP59 (SRA-1400), and rabbit polyclonal anti-Cdc37 (SPA-605) were from Assay Designs (Ann Arbor, MI); rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ab9485) was from Abcam (Cambridge, MA); goat anti-T-cell-restricted intracellular antigen (TIA) (sc-1751) was from Santa Cruz Biotechnology (Santa Cruz, CA); and mouse monoclonal anti-RAS (RAS10) was from Millipore (Billerica, MA). Donkey anti-rabbit conjugated to Alexa647 (A31573), donkey anti-mouse conjugated to Alexa488 (A21202), donkey anti-rabbit conjugated to Alexa488 (A21206), goat anti-mouse conjugated to Alexa750 (A21037), and goat anti-rabbit conjugated to Alexa750 (A21039) secondary antibodies were from Invitrogen. Donkey anti-human conjugated to Texas Red and goat anti-rabbit and goat anti-mouse conjugated to horseradish peroxidase were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Plasmids

A plasmid encoding a GFP-hAgo2 fusion under the control of a tetracycline-responsive promoter was constructed as follows. Using EagI linearized pGFP-hAgo2 (Addgene, Cambridge, MA) as a template, a polymerase chain reaction (PCR) containing the primers eGFP-hAGO2-AflII-F (TAC TCT TAA GTC GCC ACC ATG GTG AGC AAG G) and eGFP-hAGO2-XbaI-R (ACT TCT AGA TTA AGC AAA GTA CAT GGT GCG C) was used to amplify a GFP-hAgo2 fragment. The PCR product was amplified in the shuttle vector pCRII-Blunt-TOPO and then subcloned into the AflII and XbaI sites of pcDNA4/TO (Invitrogen) to produce the plasmid pcDNA4/TO-GFP-hAgo2. To generate pDsRed-hAgo2, pGFP-hAgo2 was digested with HindIII and

BamHI and ligated into pDsRed-C1-Monomer (Clontech, Mountain View, CA). A DsRed-tagged TIA-1 expression plasmid (pDsRed-Monomer-C1-TIA1) was constructed as follows. The TIA-1 cassette was removed from pEGFP-TIA1 (obtained from Dr. J. F. Cáceres, Western General Hospital, Edinburgh, Scotland, United Kingdom) by digestion with XhoI and XbaI and then ligated in-frame and downstream from the DsRed cassette in the vector pDsRed-Monomer-C1 (Clontech). GFP- and DsRed-tagged versions of TRBP were constructed as follows. Using pDest30-HA-TRBP2 (from Dr. W. Filipowicz, Friedrich Miescher Institute, Basel, Switzerland) as a template for PCR using the primers TRBP-F-XHOI (G CTC GAG CC ATG AGT GAA GAG) and TRBP-R-BAMHI (G GGA TCC CTT GCT GCC TGC CAT G), a TRBP fragment was generated. The PCR product was then ligated into the XhoI and BamHI sites of pDsRed-C1-Monomer and pEGFP. A GFP-tagged version of Dicer was constructed as follows. Using pcDNA 5TO/CFP-hDicer as a template in a PCR with the primers hDcr1-for-XhoI (GC CTC GAG GC ATG AAA AGC CCT GCT TTG CA) and hDcr1-rev-HindIII (GC AAG CTT GCT ATT GGG AAC CTG AGG TT), a hDicer fragment was generated. The PCR fragment was first cloned into pCR-BluntII-TOPO and then amplified in pBluscript, after which it was subcloned into the XhoI and HindIII sites of pEGFP. The plasmid encoding GFP-TNRC6A (GW182) was a gift from Dr. E. Chan (University of Calgary).

Cell Culture and Transfection

HeLa, human embryonic kidney 293T, and HepG2 cells and mouse embryo fibroblasts 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). For six-well plates, a total of 1 μg of plasmid DNA and 1.5 μl of transfection reagent were used per well. Stably transfected cell lines expressing GFP-hAgo2 under the control of a tetracycline-responsive promoter were generated by transfection of HeLa/TREx cells with pcDNA4/TO-GFP-hAgo2 followed by selection with Blasticidin (5 $\mu\text{g}/\text{ml}$) and Zeocin (400 $\mu\text{g}/\text{ml}$). After expansion and selection, the stable clones were cultured in DMEM containing Blasticidin and Zeocin as well as 15% heat-inactivated FBS. To induce expression of GFP-hAgo2, cells were treated with 1 $\mu\text{g}/\text{ml}$ doxycycline (Sigma-Aldrich). PACT-deficient mouse embryonic fibroblasts (Rowe *et al.*, 2006) were a gift from Dr. Ganes Sen (Cleveland Clinic). TRBP-deficient mouse embryonic fibroblasts were obtained from Dr. R. Braun (The Jackson Laboratory, Bar Harbor, ME).

Fluorescence Microscopy and Live-Cell Imaging

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

Samples were viewed on an Axiovert 200M (Carl Zeiss, Thornwood, NY) equipped with a spinning disk confocal unit (UltraView ERS) and Velocity software (PerkinElmer Life and Analytical Sciences, Boston, MA) microscope. Images were acquired with a C9100-050 EM-charge-coupled device (Hamamatsu Photonics, Hamamatsu City, Japan) digital camera. Images were quantitated using Imaris (Bitplane, St. Paul, MN) or ImageJ software (National Institutes of Health, Bethesda, MD) and compiled with Photoshop software (Adobe Systems, San Jose, CA). In all cases, samples were viewed using a Plan ApoChromat 63 $\times$ objective lens (Carl Zeiss) with a numerical aperture of 1.4.

For live-cell imaging experiments, cells were cultured in 35-mm glass-bottomed culture dishes (P35G-1.5-14-C; MatTek, Ashland, MA). Before viewing, the culture media were replaced with CO_2 -independent culture media (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum. Cells were maintained at 37°C using an objective warmer (Biopetechs, Butler, PA) and heated stage (Carl Zeiss).

Immunoblotting

Total cell lysates were prepared by adding 5 $\times$ 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 10% SDS-polyacrylamide gels, transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore), and incubated with primary antibodies diluted in 10% skim milk powder in Tris-buffered saline/Tween 20. After washing, samples were incubated with secondary antibodies that were conjugated to Alexa750 (Invitrogen) or horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA). The membranes were then exposed to an Odyssey infrared imaging system and software (Li-Cor Biosciences, Lincoln, NE) for quantitative Western blot analyses or used to expose Fujifilm, which was developed on a Kodak XO-MAT film developer, scanned using an Epson Perfection 4870 Photo scanner, and analyzed with ImageJ software.

In Vitro siRNA-directed Cleavage Assay

Drosophila ovaries were isolated from female flies (5 g) as described previously (Theurkauf *et al.*, 1992). Isolated ovaries were homogenized in 1 ml of ice-cold RNAi buffer (100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, and 2 mM magnesium acetate) containing dithiothreitol and Complete EDTA-free protease inhibitor tablets (Roche Diagnostics, Indianapolis, IN) by using a glass tissue grinder (Kontes Glass, Vineland, NJ) as described previously (Tomari *et al.*, 2004). The homogenates were clarified by centrifugation at $18,000 \times g$ for 25 min at 4°C after which the supernatant was aliquoted (50 μ l) and then flash frozen in liquid nitrogen. Aliquots were stored at -80°C before use.

A 177-nt sense luciferase target RNA was generated from pGEM-Luc (Invitrogen), between positions 113 and 273 relative to the start codon followed by a 17-nt complement of the SP6 promoter sequence (Elbashir *et al.*, 2001). The sense strand target RNA was 5'-cap labeled with vaccinia virus guanylyltransferase in the presence of [α - ^{32}P]guanosine triphosphate according to the manufacturer's (Ambion, Austin, TX) instructions. The sequence of the ds siRNA (Invitrogen) targeting the 177-nt sense RNA was as follows: 5'-OH-CGUACGCGGAUACUUCGAAA-OH-3' (sense), and 5'-OH-UCGAAGAUUCCGCGUACGUG-OH-3' (antisense).

In vitro RNAi reactions were assembled as described previously (Tuschl *et al.*, 1999), except the lysates were incubated with 25, 50, 75, or 100 μM Geld or equal volumes of DMSO (vehicle) for 30 min before reaction assembly. Samples were then subjected to autoradiography after urea (8 M) gel electrophoresis in Tris-borate buffer.

Primary Antibody Cross-linking and Coimmunoprecipitation of Hsp90–Argonaute Complexes

Anti-Hsp90 monoclonal antibodies and protein G-Sepharose 4 fast flow beads (17-0618-01; GE Healthcare, Chalfont St. Giles, Buckinghamshire, United Kingdom) were mixed briefly together in PBS for 1 h at room temperature and then washed twice with 10 volumes of 100 mM sodium borate before cross-linking with 20 mM dimethylpimelimidate for 30 min at room temperature. Samples were then washed twice with 10 volumes of 200 mM ethanolamine before further incubation for 2 h at room temperature in 200 mM ethanolamine. Finally, samples were washed three times with 10 volumes of PBS and stored as a 1:1 slurry in PBS at 4°C.

Cells were lysed in 50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM MgOAc, and 1% Triton X-100 with Roche protease inhibitors and 20 mM sodium molybdate. Cell lysates were cleared at $14,000 \times g$ for 10 min, and Hsp90 complexes were immunoprecipitated for 1 h at 4°C and then washed once with binding buffer. Beads were boiled in sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting.

Immunoprecipitation and miRNA Detection

Stably transfected HeLa/TREx were induced to express GFP-hAgo2 by the addition of doxycycline to a final concentration of 1 $\mu\text{g}/\text{ml}$. 6 h after the addition of doxycycline, cells were treated with DMSO or Geld for an additional 12 h. Lysates were prepared by lysing cells in 25 mM Tris, pH 7.5, 150 mM NaCl, 0.5% NP40, supplemented with Complete EDTA-free protease inhibitor (Roche Diagnostics) on ice for 10 min. Lysates were clarified by centrifugation at $10,000 \times g$ for 10 min at 4°C. Immunoprecipitation of GFP-tagged hAgo2 was performed using goat anti-GFP antibody for 2 h, with end-over-end rotation, at 4°C. Protein G-Sepharose 4 Fast Flow (GE Healthcare) beads were blocked with 1% casein in lysis buffer for 2 h at 4°C and then added to samples for an additional 1 h at 4°C with rotation. Aliquots of bound fractions were taken for protein analysis, and the remainder was subjected to RNA isolation using TRIzol Reagent (Invitrogen) following the manufacturer's protocol. Reverse transcription was performed using let-7c RT Primer (AM30002; Ambion) and qScript Flex cDNA kit (Quanta Biosciences, Gaithersburg, MD). Quantitative PCR was conducted on Mx3005P real-time PCR machine (Stratagene, La Jolla, CA) by using let-7c PCR primer set (Ambion) and PerfeCTa SYBR Green SuperMix, UNG, Low Rox reagent (Quanta Biosciences).

RESULTS

Previous studies by our laboratory and other groups have revealed that Argonaute proteins bind to the molecular chaperone Hsp90 (Tahbaz *et al.*, 2001, 2004; Maniataki and Mourelatos, 2005; Hock *et al.*, 2007); however, the specific requirement for this chaperone in Argonaute function has not been examined. Unlike most other chaperones that assist in folding of a vast array of nascent polypeptides, Hsp90 has a restricted set of client proteins, many of which are involved in signal transduction (Caplan, 1999). The precise mechanism by which Hsp90 "matures" client proteins is not known, but it is well established that this chaperone can

affect gene expression by modulating nuclear translocation of transcription factors (Pratt, 1993; Smith, 1993). To determine whether Hsp90 activity is required for targeting of hAgo2 to RNA granules that function in gene-silencing, we used live-cell imaging experiments. First, we constructed GFP- and DsRED-labeled hAgo2 expression plasmids for use in transient and stably transfected mammalian cells. Similar to previously published studies (Jakymiw *et al.*, 2005; Liu *et al.*, 2005), fluorescent hAgo2 was distributed between the cytoplasm and discrete cytoplasmic foci that contained the PB marker GW182 (Figure 1). In control cells, there was no overlap between hAgo2 and the SG marker TIA; however, treatment of cells with the translational inhibitor hippuristanol (Bordeleau *et al.*, 2006; Leung *et al.*, 2006) resulted in rapid recruitment of GFP-hAgo2 and DsRed-TIA to nascent SGs (Figure 1).

We also examined localization of the hAgo2-binding proteins Dicer, TRBP, and PACT in control and stressed cells. These proteins form a large complex that facilitates maturation of miRNAs and RISC assembly (Maniataki and Mourelatos, 2005; Lee *et al.*, 2006). None of the Argonaute-binding proteins was associated with cytoplasmic foci in control cells, but instead, they localized diffusely throughout the cytoplasm and nucleus (Figure 2). We attribute the nuclear staining with anti-Dicer and anti-TRBP reagents to nonspecific binding, because when the experiments were repeated with fluorescently tagged Dicer and TRBP, it was observed that both proteins were largely confined to the cytoplasm (Supplemental Figure 1). Similar results were reported by other laboratories (Provost *et al.*, 2002; Laraki *et al.*, 2008). In contrast to Dicer and TRBP, PACT was efficiently recruited to TIA-positive SGs (Figure 2), indicating that components of the RISC loading complex react differently to translational stress. We used a PACT-deficient cell line to determine whether this protein was essential for biogenesis of SGs or PBs. However, neither PACT nor TRBP is required for biogenesis of PBs or SGs (Supplemental Figure 2). Similarly, recruitment of Argonaute proteins to SGs is not dependent on these proteins.

Next, we investigated whether Hsp90 activity was required for recruitment of hAgo2 to SGs and PBs. HeLa cells expressing GFP-hAgo2 were treated with the Hsp90 inhibitor Geld or DMSO alone, and then SG formation was induced with hippuristanol. Under control conditions, SG formation occurred minutes after cells were exposed to hippuristanol (Figure 3A and Supplemental Movies). TIA is considered a nucleating factor in SG formation (Anderson and Kedersha, 2008); as such, coalescence of GFP-TIA into nascent SGs was used to monitor their biogenesis. Preincubation of hippuristanol-treated cells with Geld did not dramatically affect the recruitment of GFP-TIA to SGs, indicating that Hsp90 activity is not required for assembly of SGs (Figure 3, Supplemental Figure 3, and Supplemental Movies). However, this drug seemed to reduce the rate by which GFP-hAgo2 was targeted to SGs (Figure 3, A and B, and Supplemental Movies). Quantitation of the data confirmed that compared with control cells, Geld treatment substantially reduced the total numbers of GFP-hAgo2-positive SGs as well as the average fluorescence intensity of the individual granules (Figure 3B). Unlike hAgo2, Geld treatment did not significantly affect the recruitment of PACT to SGs (Figure 4). Together, these data indicate that Hsp90 activity is important for recruitment of Ago2 to SGs but not for formation of these structures.

Figure 1. HeLa cells transiently expressing GFP-GW182, GFP-hAgo2, DsRed-hAgo2, or DsRed-TIA were analyzed by live-cell confocal microscopy techniques 24 h after transfection. Images were acquired immediately before addition of 1 μ M hippuristanol (–) or 20 min after addition of the drug (+). Arrowheads indicate the presence of GFP-hAgo2 in PBs of control cells. Hippuristanol treatment results in rapid translocation of GFP-hAgo2 and DsRed-TIA to nascent SGs (arrows). Insets show enlarged regions that are indicated by white boxes. Bar, 10 μ m.

Hsp90 and a Subset of Cochaperones Colocalize with hAgo2 and TIA in Stress Granules

To determine whether Hsp90 complex components are themselves recruited to SGs, PBs, or both, we examined the relative localizations of Hsp90 and associated cochaperones under control and stressed conditions. In control cells, Hsp90 was homogeneously distributed throughout the cytoplasm and nuclei. However, when translational stress was induced, a pool of Hsp90 was recruited to newly formed SGs (Figure 5A, arrowheads). Similarly, the early and late Hsp90 cochaperones Hop and Aha1, respectively (Figure 5, A and B), were also recruited to nascent Ago2-positive SGs. There are two main types of Hsp90 complexes that are defined by the presence of specific cochaperones (Caplan, 1999). Maturation of some protein kinases requires the cochaperone Cdc37, whereas Hsp90 complexes that regulate maturation and transport of steroid hormone receptors contain immunophilins such as FKBP59. In Figure 5A and Supplemental Figure 4, it can be seen that Cdc37 but not FKBP59 is recruited to SGs. The ubiquitous cochaperone p23 is also recruited to SGs (Supplemental Figure 4). Although correlative, these observations are consistent with the possibility that a kinase-specific Hsp90 complex is recruited to SGs during translational stress.

We next asked whether colocalization between hAgo2 and Hsp90 complex components resulted from increased association between hAgo2 and Hsp90. Lysates prepared from

hippuristanol-treated and control cells were subjected to coimmunoprecipitation and immunoblot analysis to determine the relative binding between Hsp90 and Argonaute proteins. Surprisingly, hippuristanol had no appreciable effect on the amount of Hsp90-associated Ago2 (Figure 5C). Similarly, inhibition of Hsp90 activity did not significantly affect the binding of Argonautes to Hsp90.

Hsp90 Activity Is Important for Integrity of PBs

A recent study reported that Argonaute proteins associate with SGs and PBs with different kinetics (Leung *et al.*, 2006). Specifically, whereas Argonautes are stably associated with PBs, the SG-associated pool exchanges rapidly with the cytosolic pool. These results suggest that interaction of Argonautes with PBs and SGs is governed by different factors. To determine whether Hsp90 activity is also required for association of hAgo2 with PBs, we performed live-cell imaging in HeLa cells expressing hAgo2. Our strategy of choice was to use fluorescence recovery after photobleaching to monitor the exchange of cytoplasmic GFP-hAgo2 with PB-associated GFP-hAgo2; however, because PBs are highly motile, it was difficult to completely bleach GFP-hAgo2 from PBs without quenching part of the cytoplasmic pool of GFP-hAgo2 (data not shown). Therefore, we constructed stably transfected HeLa cells expressing GFP-hAgo2 under the control of an inducible promoter. These cells were then used to monitor the recruitment of nascent GFP-hAgo2 to P-bodies.

Figure 2. PACT, but not Dicer or TRBP, is recruited to nascent SGs. HeLa cells were treated with hippuristanol (1 μ M) or DMSO for 60 min before fixation and processing for indirect immunofluorescence. Nascent PACT-positive SGs are indicated (arrows). Bar, 10 μ m.

Figure 6, A and B, shows that in Geld-treated cells, association of GFP-hAgo2 with PBs is virtually abrogated. It is important to point out that the numbers of GFP-hAgo2-positive PBs in control cells (2–4 PBs/cell) are lower than in fixed cells stained with anti-GW182 antibodies (Figure 6C). This may reflect the fact that targeting of nascent GFP-hAgo2 to PBs requires exchange with the pool of unlabeled Argonaute proteins that stably associate with PBs (Leung *et al.*, 2006). Alternatively, it is possible that PBs are heterogeneous and that only a subpopulation of these RNPs contain hAgo2. Induction of GFP-hAgo2 expression with doxycycline was generally less robust in cells treated with Geld (Figure 6, A and B), and this may be due to nascent Ago2 being less stable in the absence of Hsp90 activity (Tahbaz *et al.*, 2001). Similar results have been reported for other Hsp90 client proteins (Whitesell *et al.*, 1994).

To address the potential scenario that PB integrity is dependent on Hsp90 activity, HeLa cells were treated with or without Geld for 10 h, and the numbers of PBs were determined using antibodies to GW182, a core component of these RNPs. Results in Figure 6C show that inhibition of Hsp90 activity for 10 h results in a complete loss of microscopically visible PBs. These data indicate that in contrast to

SGs, the biogenesis, stability, or both of PBs require Hsp90 activity.

Inhibition of Hsp90 Activity Affects siRNA- and miRNA-dependent Functions of Argonautes

The results described above, together with recent work suggesting that PBs are formed as a consequence of RNA-mediated silencing (Eulalio *et al.*, 2007), led us to speculate that Hsp90 activity is also required for Argonaute function in miRNA-mediated gene silencing, siRNA-mediated gene silencing, or both. We first investigated the effect of Geld treatment on miRNA-mediated silencing. The miRNA function assay used in this study is based upon the levels of Ras protein being regulated by the let-7c miRNA (Johnson *et al.*, 2005). Specifically, in a given cell, Ras levels are inversely proportional to the levels of let-7c miRNA. Accordingly, we predicted that if Geld inhibits the ability of Argonautes to bind let-7c, or function after binding let-7c, levels of Ras protein would increase in drug-treated cells. Indeed, levels of Ras protein were, on average, nearly 45% higher in Geld-treated HeLa cells. These data are comparable with those of Johnson *et al.* (2005) who reported that inhibition of let-7c increases Ras protein levels by 60–70%. The steady-state levels of Argonaute proteins were similar in control and Geld-treated samples (Figure 7, A and B), which indicates that drug-induced decrease in Argonaute protein concentration is not the reason for elevated Ras expression. We also determined whether other putative miRNA targets were affected when Hsp90 activity was inhibited. Specifically, we observed that levels of cytochrome *c* protein were significantly increased in Geld-treated cells (Supplemental Figure 5). Using web-based algorithms (<http://microrna.sanger.ac.uk/>), we determined that cytochrome *c* is a high confidence target of mi29-b, an miRNA that like let-7c, is highly expressed in HeLa cells (Yeung *et al.*, 2005). Finally, we also monitored Ras protein levels in Geld-treated HepG2 cells. This cell line contains low levels of let-7c miRNA (Johnson *et al.*, 2005), and as predicted, Ras levels were not significantly affected by this drug (Figure 7, A and B).

We next assessed whether siRNA-directed cleavage of mRNA was affected by Geld. For this assay, *Drosophila* ovary extracts were programmed with 5' cap-labeled single strand luciferase target RNA and a luciferase-specific siRNA (Tomari *et al.*, 2004). Figure 7C shows that Geld inhibits the siRNA-directed cleavage of [32 P]luciferase RNA in a dose-dependent manner. Together, the data from these experiments indicate that miRNA-dependent translational repression and siRNA-directed cleavage functions of Argonaute proteins are dependent upon Hsp90 activity.

Although Argonaute complexes can be programmed with small RNAs that do not require Dicer processing (Elbashir *et al.*, 2001), several recent studies suggest that when coupled with Dicer activity, RISC activation is much more efficient (Pham *et al.*, 2004; Chendrimada *et al.*, 2005; Gregory *et al.*, 2005; Maniataki and Mourelatos, 2005). Given that formation of stable Dicer/Argonaute complexes is dependent on Hsp90 activity (Tahbaz *et al.*, 2004), we next questioned whether this chaperone is also required for loading of small RNAs onto Argonaute. Therefore, we compared the amount of let-7 miRNA-associated with GFP-hAgo2 in cells that had been treated with or without Geld. Expression of GFP-hAgo2 was induced for 6 h in stably transfected cells before introduction of Geld. Induction of GFP-hAgo2 and inhibition of Hsp90 was continued for an additional 12 h, after which immunoprecipitations were performed using anti-GFP antibodies. RNA was isolated from load, flow-through, and bound fractions, and levels of let-7c miRNA were ana-

Figure 3. Geld inhibits recruitment of GFP-hAgo2 to SGs. HeLa cells were transiently transfected with plasmids encoding GFP-TIA or GFP-hAgo2. Twenty-four hours after transfection, cells were treated with DMSO (–) or 10 μ M Geld (+) for 6 h after which hippuristanol was added to a final concentration of 1 μ M. Cells were then imaged every 30 s for a total of 20 min. (A) Still images are presented from beginning, mid-, and end time points. The insets in the bottom left of the panels are enlargements of the areas within the squares. Bars, 10 μ m. (B) Fluorescent intensities of newly forming SGs in Geld or DMSO (control)-treated samples were measured at regular intervals after addition of hippuristanol. Data were acquired using Imaris software, and the results are plotted. Structures identified as SGs were required to meet or exceed a minimum size and fall within a defined range of fluorescent intensities. These parameters were determined by analysis of control cells and held constant for all samples of a given GFP-construct. Importantly, for GFP-hAgo2, the range of fluorescent intensity excluded existing GFP-hAgo2-positive PBs, because these structures were invariably brighter than newly forming SGs.

Figure 4. Geld treatment does not inhibit recruitment of PACT to SGs. Cells were pretreated with Geld (10 μ M) or DMSO for 6 h before addition of hippuristanol (500 nM). After 30 min, the cells were processed for indirect immunofluorescence and images were acquired using a spinning disk confocal microscope. Newly formed SGs that contain PACT are indicated by arrowheads. Bar, 10 μ m.

lyzed by quantitative PCR. Figure 7D shows that levels of let-7c miRNA associated with GFP-hAgo2 were unaffected by Geld treatment. Furthermore, the total level of let-7c miRNA remains unchanged after 12 h of Geld treatment. Because mature miRNAs are very stable (Lund *et al.*, 2004), it is possible that significantly longer drug treatment times may be required to observe effects on miRNAs levels. However, prolonged treatment with Geld can lead to cell cycle arrest and cytotoxicity in cultured cells.

DISCUSSION

The RNAi apparatus modulates the expression of potentially thousands of genes in mammalian cells; and not sur-

prisingly, recent evidence suggests that components of this pathway are themselves subject to regulation. One means by which gene-regulatory networks are controlled is through spatial-temporal localization of their components. In this regard, some Argonaute functions are thought to occur in the context of cytoplasmic ribonucleoprotein complexes that include PBs (Liu *et al.*, 2005; Sen and Blau, 2005) and SGs (Leung *et al.*, 2006). Data from the present study suggest that the specialized chaperone Hsp90 plays a critical role in regulating the association of Argonautes with SGs as well as the assembly/stability of PBs. Although minimal RISC and RISC loading complexes can spontaneously assemble in vitro (Rivas *et al.*, 2005; MacRae *et al.*, 2008), based on analogy with other RNPs (Yong *et al.*, 2004; Matera *et al.*, 2007), it is likely that formation of these structures is tightly controlled in vivo. Indeed, during the preparation of the present work, it was reported that Hsp90 regulates the assembly of L7Ae-containing RNPs in the nucleus (Boulon *et al.*, 2008) and that biogenesis of PBs and SGs is governed by >100 genes (Ohn *et al.*, 2008). These observations are consistent with previous studies that showed that the size and numbers of PBs fluctuates in a cell-cycle-dependent manner (Yang *et al.*, 2004; Carmichael *et al.*, 2006). Because Hsp90 is essential for viability (Bohen and Yamamoto, 1993), it would not have been picked up as a PB assembly factor in the genetic screen (Ohn *et al.*, 2008).

Although PBs and SGs share several common components, the RNAi screen Ohn *et al.* (2008) clearly demonstrates that the assembly/stability of these RNPs is governed by different factors. Further illustrating this point is the observation that although Hsp90 activity is not critical for SG assembly per se, this chaperone and many of its cofactors, including Hop, Cdc37, and Aha1, which did not colocalize with PBs under any condition tested, are recruited to SGs during translational stress. Only one kinase, dual-specificity tyrosine phosphorylation-related kinase 2 is known to be important for SG assembly (Ohn *et al.*, 2008), but this enzyme is not an Hsp90 client (Richter *et al.*, 2007). As such, the significance of Hsp90 recruitment to nascent SGs remains to be determined.

Our data are consistent with a scenario in which hAgo2 and potentially other Argonautes require Hsp90 activity for their functions in siRNA- and miRNA-dependent gene-silencing pathways. There are several possible mechanisms by which Hsp90 could regulate the function of Argonautes and their targeting to RNA granules. Cellular kinases are well known clients of Hsp90 (reviewed in Pratt and Toft, 2003; Wegele *et al.*, 2004) and given that phosphorylation of hAgo2 is reportedly important for targeting to PBs (Zeng *et al.*, 2008), we considered the possibility that Geld interferes with the activity of an Argonaute-specific kinase. However, this seems unlikely because unlike many tyrosine kinases that are inhibited by Geld, this drug seems to activate the p38 mitogen-activated protein kinase pathway (Dey and Cedarbaum, 2007), which is involved in phosphorylation of hAgo2. Accordingly, we favor the hypothesis that hAgo2, and presumably other Argonaute proteins, are bona fide clients of Hsp90. Because the binding site for Geld overlaps with the ATP binding site in Hsp90 (Smith *et al.*, 1995), it is likely that Hsp90-dependent maturation of Argonautes requires ATP binding. In this regard, it is interesting to note that although ATP hydrolysis is not required for RISC activity, ATP does stimulate RISC activity by two- to threefold (Gregory *et al.*, 2005). If substoichiometric amounts of Hsp90 were present in the RISC preparations described by Gregory *et al.* (2005), it is possible that the ATP-dependent increase in RISC activity was due the chaperone activity of Hsp90.

Figure 5. Hsp90 and kinase-specific cochaperones localize to nascent SGs. (A) HeLa cells were treated with DMSO (–) or 1 μ M hippuristanol (+) for 1 h before processing for indirect immunofluorescence by using antibodies specific for Hsp90, Aha1, Cdc37, and FKBP59. Newly formed SGs are marked with arrowheads. (B) Control or stably transfected HeLa cells expressing GFP-hAgo2 were treated with 1 μ M hippuristanol for 1 h. Cells were then processed for indirect immunofluorescence using antibodies specific for Hsp90, Aha1, Cdc37, hAgo2, or HOP. The inset panels in the bottom right are enlargements of the areas marked by white squares. Bars, 10 μ m. (C) HeLa cells DMSO (Control), 1 μ M hippuristanol (+HIPP), 10 μ M Geld (+Geld), or both drugs (+Geld; +HIPP) were subjected to immunoprecipitation with anti-Hsp90 followed by immunoblotting with antibodies to Hsp90 and hAgo2.

Figure 6. Effect of Geld on P-bodies. (A) Expression of GFP-hAgo2 was induced in stably transfected HeLa cells by addition of doxycycline (+DOX) for 6–10 h in the presence or absence of Geld (Geld or DMSO, respectively). Images were acquired immediately before induction with DOX and also at 6 and 10 h after induction. The insets in the bottom left are enlargements of the areas within the white squares. (B) Average numbers of GFP-hAgo2-positive PBs in DMSO- and Geld-treated cells were determined. In addition, the average fluorescent intensities of GFP-hAgo2 in the cytoplasm at 6 and 10 h after induction with DOX are shown (arbitrary units). (C) HeLa cells were treated with DMSO and Geld for 6 and 10 h after which they were processed for indirect immunofluorescence by using antibodies specific for the PB resident protein GW182. Bars, 10 μ m.

Previously, we reported that Hsp90 activity is important for stable interaction between Argonaute proteins and Dicer (Tahbaz *et al.*, 2004). Because Dicer is part of the RISC loading complex, we hypothesized that loading of miRNAs onto hAgo2 would be affected by Geld treatment. We did not find evidence to support this theory, but it is important to realize that although molecular chaperones often increase the efficiency of specific processes, in many cases they are not absolutely required for these processes to occur. For example, calnexin and BiP facilitate the assembly of major histocompatibility complex I and immunoglobulin G complexes, respectively, but both processes can occur, albeit less efficiently, in the absence of these chaperones (Bole *et al.*, 1986; Jackson *et al.*, 1994). In this respect, Argonaute proteins can reportedly bind to small RNAs *in vitro* and *in vivo* without the RISC loading complex (Rivas *et al.*, 2005; Jagannath and Wood, 2009). Based on these considerations, we propose that Hsp90 is not absolutely essential for RISC loading but instead may accelerate or improve the efficiency of this process by promoting or stabilizing interactions between Argonautes and Dicer. Under this scenario, the extended incubation times with Geld may allow RISC loading to overcome the kinetic barrier that is normally lowered by Hsp90. An alternative but not

mutually exclusive possibility is that in the absence of Hsp90 activity, mi- and siRNAs are loaded onto Argonautes normally; however, the resulting RISCs have lower activity due to a defect in maturation of the Argonaute proteins.

The loss of PBs in Geld-treated cells may also be related to Argonaute dysfunction. Although microscopic PBs are not required for small RNA-dependent gene silencing (Chu and Rana, 2006; Eulalio *et al.*, 2007), the formation, stability, or both, of these RNPs are dependent on a functional RNAi pathway (Pauley *et al.*, 2006; Lian *et al.*, 2007). Furthermore, decreased expression of hAgo2 is associated with reduced numbers of these structures. Accordingly, if Ago1, Ago3, and Ago4 have redundant functions in PB biogenesis/stability, the complete loss of PBs in Geld-treated cells may reflect the requirement for Hsp90 in maturation of all Argonaute proteins.

Finally, this study provides relevant new information about three important Argonaute-binding proteins—Dicer, TRBP, and PACT—that function in RNA-dependent gene silencing (Chendrimada *et al.*, 2005; Haase *et al.*, 2005; Lee *et al.*, 2006). Together with hAgo2, a pool of these proteins exists in a 500-kDa complex that functions in RISC assembly/activation; however, our data are also consistent with a

Figure 7. Inhibition of Hsp90 impairs Argonaute-dependent posttranscriptional gene silencing. (A) HeLa and HepG2 cells were treated with DMSO or 10 μ M Geld for 12 h. The level of endogenous Ras is shown with GAPDH provided as a loading control. Levels of endogenous Argonaute proteins in HeLa cells were determined using a PAZ-domain specific polyclonal antibody. (B) Quantitation of the level of Ras, normalized to GAPDH and relative to DMSO control, is shown for HeLa and HepG2 samples. (C) *Drosophila* ovary extracts were incubated with 32 P end-labeled target RNA (Target) with or without homologous siRNA, DMSO, or Geld. After incubation at 25°C for 30 min, samples were subjected to denaturing gel electrophoresis and autoradiography. The positions of the Target and cleavage product (Product) are shown. (D) Cells expressing GFP-hAgo2 were treated with DMSO or 10 μ M Geld for 12 h after which time total RNA was isolated and GFP-hAgo2 was immunoprecipitated from the lysates. The levels of total and GFP-hAgo2 associated let-7 miRNA were determined by quantitative RT-PCR.

scenario in which PACT and hAgo2 form a separate SG-specific complex. This is intriguing because although TRBP and PACT have overlapping functions in RNAi pathways, only PACT is critical for biogenesis, stability, or both of miRNAs (Haase *et al.*, 2005; Lee *et al.*, 2006). Whether PACT is critical for the function of Argonaute proteins in SGs during cellular stress or vice versa remains to be determined. However, that PACT is not required for biogenesis of PBs or SGs nor for recruitment of Argonaute proteins to SGs is consistent with our hypothesis that the negative effects of Geld on small RNA-dependent gene silencing are the result of compromised Argonaute function.

ACKNOWLEDGMENTS

We thank Dr. A. Simmonds (University of Alberta) for providing advice for the live-cell imaging experiments. This work was funded by grants from the Canadian Institutes of Health Research (CIHR; to T.C.H.) and National Institutes of Child Health and Development grant R01-HD036631 (to P. L.). J.M.P. is the recipient of a Doctoral Scholarship from CIHR. P. L. is a James McGill Professor, and T.C.H. is the recipient of a Medical Scientist award from the Alberta Heritage Foundation for Medical Research. N. T. is the recipient of a postdoctoral award from CIHR.

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