

Hsp90 cochaperones p23 and FKBP4 physically interact with hAgo2 and activate RNA interference–mediated silencing in mammalian cells

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ABSTRACT Argonaute proteins and small RNAs together form the RNA-induced silencing complex (RISC), the central effector of RNA interference (RNAi). The molecular chaperone Hsp90 is required for the critical step of loading small RNAs onto Argonaute proteins. Here we show that the Hsp90 cochaperones Cdc37, Aha1, FKBP4, and p23 are required for efficient RNAi. Whereas FKBP4 and p23 form a stable complex with hAgo2, the function of Cdc37 in RNAi appears to be indirect and may indicate that two or more Hsp90 complexes are involved. Our data also suggest that p23 and FKBP4 interact with hAgo2 before small RNA loading and that RISC loading takes place in the cytoplasm rather than in association with RNA granules. Given the requirement for p23 and FKBP4 for efficient RNAi and that these cochaperones bind to hAgo2, we predict that loading of hAgo2 is analogous to Hsp90-mediated steroid hormone receptor activation. To this end, we outline a model in which FKBP4, p23, and Aha1 cooperatively regulate the progression of hAgo2 through the chaperone cycle. Finally, we propose that hAgo2 and RNAi can serve as a robust model system for continued investigation into the Hsp90 chaperone cycle.

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INTRODUCTION

Posttranscriptional gene-silencing pathways are conserved throughout eukaryotes. In mammalian cells, it is estimated that the effector proteins of these pathways regulate 60% of genes at the posttranscriptional level (Lewis *et al.*, 2005; Friedman *et al.*, 2009). Therefore these proteins directly or indirectly affect the majority of cellular pathways. MicroRNAs (miRNAs) and small interfering RNAs (siRNAs) provide specificity in posttranscriptional gene-silencing pathways. These small RNAs are derived from hairpin-forming

transcripts or long double-stranded RNAs through a series of endonuclease-mediated cleavages (Ender and Meister, 2010). The final cleavage step and subsequent incorporation of small RNAs into Argonaute-containing ribonucleoprotein complexes are mediated by Dicer (Bernstein *et al.*, 2001; Hutvagner *et al.*, 2001; Ketting *et al.*, 2001). After being loaded onto Argonaute, miRNA duplexes are unwound and a single strand (“guide”) remains associated with the complex, whereas the other (“passenger”) is removed. Argonaute proteins, together with a guide RNA, form the minimal RNA-induced silencing complex (RISC; Hammond *et al.*, 2000; Martinez *et al.*, 2002).

RISC is targeted by base pairing between the guide strand and mRNA, and the extent of sequence complementarity determines the means of translation repression. If the small RNA binds the mRNA with perfect complementarity, the endonuclease activity of the Argonaute protein cleaves the mRNA (hereafter referred to as canonical RNA interference [RNAi]; Elbashir *et al.*, 2001a,b; Schwarz *et al.*, 2002). In most cases, however, miRNAs do not form perfectly complementary duplexes with target mRNAs, and, as a result, endonucleolytic cleavage of the mRNA by Argonaute does not occur (Tolia and Joshua-Tor, 2007; Wang *et al.*, 2008a,b). Instead, translation is repressed by additional protein factors that are recruited to multiple miRNA-binding

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Abbreviations used: ATP, adenosine triphosphate; FKBP4, FK506-binding protein 4; GFP, green fluorescent protein; IgG, immunoglobulin G; miRNAs, microRNAs; PAZ, PIWI-Argonaute-Zwille; PBS, phosphate-buffered saline; PPIase, peptidyl-prolyl isomerase; RISC, RNA-induced silencing complex; RNAi, RNA interference; shRNA, short hairpin RNA; siRNAs, short interfering RNAs; TRBP, human immunodeficiency virus transactivating response RNA-binding protein; UTR, untranslated region; VSV, vesicular stomatitis virus.

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sites in the 3' untranslated region (UTR) of the mRNA. The mechanism of cleavage-independent silencing is unclear, but evidence suggests that initiation and/or elongation of translation by the ribosome are inhibited, ultimately followed by deadenylation and mRNA destabilization (Fabian *et al.*, 2010; Djuranovic *et al.*, 2011). There are four Argonaute proteins in humans, but only Argonaute2 (hAgo2) cleaves targeted mRNAs (Liu *et al.*, 2004; Meister *et al.*, 2004).

Recent data indicate that the activity of the molecular chaperone Hsp90 is involved in loading small RNAs onto Argonaute proteins (Iki *et al.*, 2010; Iwasaki *et al.*, 2010; Miyoshi *et al.*, 2010). Hsp90 binds primarily to folded client proteins and elicits a conformational change that serves to activate them (Taipale *et al.*, 2010). Client activation is achieved through a series of steps, including the binding and hydrolysis of ATP, collectively referred to as the chaperone cycle. In budding yeast, and likely other organisms, 20% of the proteome are clients of Hsp90 (Breitkreutz *et al.*, 2008). This large diversity of clients is believed to demand a high level of modularity from Hsp90, a function that is imparted by at least 20 Hsp90-binding proteins known as cochaperones (Taipale *et al.*, 2010). Cochaperones regulate multiple aspects of the Hsp90 chaperone cycle, including coordinating the interaction between Hsp90 and other chaperone systems, conferring specificity for clients (e.g., Cdc37), altering ATP binding, stimulating or inhibiting ATPase activity (e.g., Aha1 and p23, respectively), and catalyzing peptidyl-prolyl *cis/trans* isomerization of client proteins (e.g., FKBP4). By influencing these aspects of Hsp90 activity, cochaperones control the progression of clients through the chaperone cycle.

Here we report that the Hsp90 cochaperones Aha1, Cdc37, FKBP4, and p23 play important roles in RNAi. Two of these cochaperones (FKBP4 and p23) form stable complexes with Hsp90 and hAgo2, and our data suggest that this interaction occurs before binding small RNAs. We also show that Aha1 and Cdc37 are required for efficient RNAi, despite not being found in stable complex with hAgo2. We were unable to rule out the possibility that Aha1 interacts transiently with the Hsp90–hAgo2 complex; consistent with our results, however, Cdc37 has not been identified in complex with any nonkinase clients of Hsp90. These data indicate that one or more additional Hsp90 clients, matured by a chaperone complex involving Cdc37, may be required for RISC activation. By further elucidating the roles of Hsp90 cochaperones in RNAi, our findings provide new insight into the Hsp90 chaperone cycle.

RESULTS

Hsp90 and a cohort of cochaperones form a complex with hAgo2

Previous studies from our laboratory and others indicate that Hsp90 is required for Argonaute activity in RNAi pathways (Pare *et al.*, 2009; Iki *et al.*, 2010; Iwasaki *et al.*, 2010; Miyoshi *et al.*, 2010; Earley and Poethig, 2011). Given that many cochaperones regulate different aspects of the Hsp90 chaperone cycle, we expect that a distinct cohort of these cofactors participate in hAgo2 maturation. As a first step toward understanding how Hsp90 cochaperones functionally interact with hAgo2, we sought to identify those that physically interact with hAgo2. We focused on Aha1, p23, and Cdc37, which were previously shown to colocalize with hAgo2 in cytoplasmic RNA granules (Pare *et al.*, 2009). Because FKBP4 did not colocalize with hAgo2 in RNA granules, we reasoned that this cochaperone would serve as a negative control for the pull-down experiments.

We used exogenously expressed myc-tagged hAgo2 (myc-hAgo2) to ensure high level of expression and robust immunoprecipitation. This strategy also afforded us the flexibility to examine how different RNAi-defective hAgo2 mutants interact with Hsp90

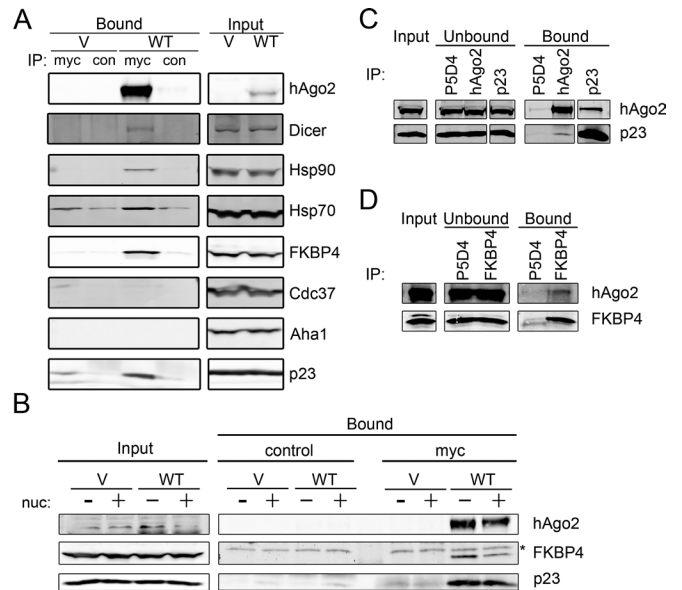


FIGURE 1: Human Argonaute2 exists in complex with Dicer, Hsp90, and the cochaperones FKBP4 and p23. (A) HeLa cells transiently transfected with pcDNA3/myc-hAgo2 (WT) or empty pcDNA3 (V) were lysed 16 h posttransfection and subjected to immunoprecipitation (IP) using Sepharose beads coated with monoclonal anti-myc antibodies (myc) or anti-VSVG (con) as a control. Total cell lysate (Input) and bound fractions were subjected to SDS–PAGE and immunoblotted for hAgo2, Dicer, Hsp90, Hsp70, and cochaperones as indicated. (B) Immunoprecipitations performed as in A were treated with or without the nuclease Benzonase (nuc) for 15 min before addition of Sepharose beads. (C, D) Lysates of untransfected HeLa cells were subjected to immunoprecipitation using monoclonal antibodies against hAgo2, p23, FKBP4, or VSV-G (P5D4) as a control. Total cell lysate (Input) and unbound and bound fractions were subjected to SDS–PAGE and immunoblotted for hAgo2, p23, and FKBP4 as indicated. Nonspecific band marked with an asterisk.

complexes. Myc-hAgo2 was immunoprecipitated from lysates of transiently transfected HeLa cells using Sepharose beads conjugated to either anti-myc or an isotype-matched immunoglobulin G (IgG) as a control. Immunoblot analysis identified Hsp90 in complex with myc-hAgo2, as previously reported (Tahbaz *et al.*, 2004; Pare *et al.*, 2009). Consistent with our previous data showing colocalization at stress granules, Hsp70 and p23 were found to coimmunoprecipitate with myc-hAgo2 (Figure 1A). Unexpectedly, we observed that FKBP4 also formed a stable complex with myc-hAgo2 but not Cdc37 or Aha1. By treating the lysates with the highly processive endonuclease Benzonase before immunoprecipitation, we demonstrated that the interactions are independent of nucleic acid (Figure 1B). Finally, we confirmed that stable interactions occur between endogenous hAgo2 and p23 (Figure 1C) and endogenous FKBP4 (Figure 1D). Because hAgo2, Hsp90, p23, and FKBP4 form a stable complex, we predicted that depletion of these cochaperones would have a significant effect on the maturation of RISC and RNAi activity.

Knockdown of specific Hsp90 cochaperones reduces efficiency of RNAi

To determine which cochaperones are required for hAgo2 function, RNAi activity assays were performed in conjunction with short hairpin RNA (shRNA)–mediated knockdown of FKBP4, Cdc37, Aha1, and p23. RNAi efficiency was assayed using a green fluorescent

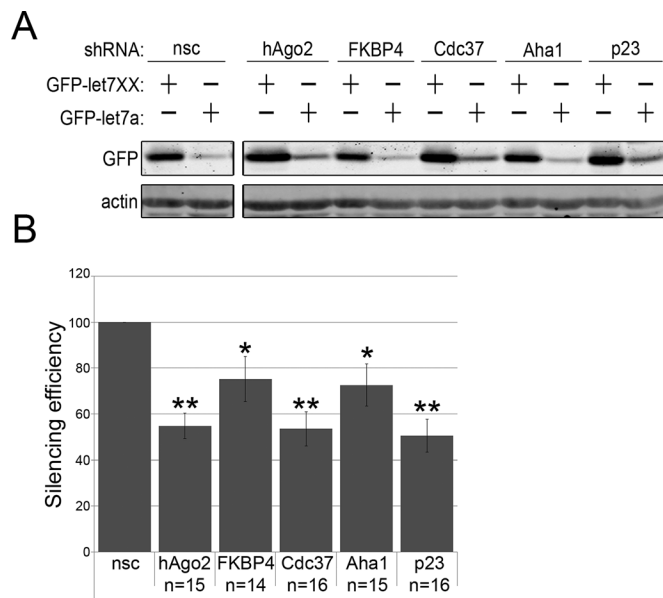


FIGURE 2: Knockdown of Hsp90 cochaperones reduces efficiency of RNAi. (A) HeLa cells were harvested 72 h after transient cotransfection with pLKO.1 vectors expressing shRNAs against hAgo2, FKBP4, Cdc37, Aha1, p23, or a nonsilencing control (nsc) as a control and a GFP-based reporter for RNAi activity (GFP-let7a) or the control (GFP-let7XX). Lysates were subjected to SDS-PAGE and immunoblotted for GFP and actin as a loading control. (B) GFP expression was quantitated using Odyssey software and normalized to actin. RNAi efficiency was calculated by determining the relative GFP expression between cells expressing GFP-let7a or GFP-let7XX. The efficiency of RNAi-mediated silencing was calculated independently for each shRNA, and the pLKO.1-nsc-transfected sample was set to 100%. Error bars represent SE, and *n* values are indicated below the axis. **p* < 0.05; ***p* < 0.01.

protein (GFP)-based reporter system (GFP-let7a) in which the 3' UTR of the mRNA contains a sequence that is perfectly complementary to the highly expressed, endogenous miRNA let7a (Khong *et al.*, 2012), thereby targeting it for repression by the canonical RNAi pathway. Replacing the let7a-binding site with a cassette (GFP-let7XX) containing mutations at positions 10 and 11, thereby preventing perfect base pairing with the let7a miRNA and inhibiting cleavage by hAgo2, alleviated repression of GFP expression (Figure 2, A and B). This is consistent with the idea that silencing of the GFP-let7a reporter relies on RISC-mediated endonucleolytic cleavage of the mRNA. The GFP-let7XX reporter was then used to control for cleavage-independent effects on the transcription, translation, folding, and stability of GFP.

HeLa cells were transiently cotransfected with the reporter GFP-let7a or GFP-let7XX and plasmids encoding shRNAs directed against hAgo2, FKBP4, Cdc37, Aha1, p23, or a nonsilencing control (nsc) hairpin that does not target mammalian genes. This strategy of simultaneous transfection eliminated potential effects of cochaperone knockdown on transfection efficiency that would alter GFP expression levels. After 24 h, cells were incubated in media containing puromycin to enrich for cells bearing shRNA-expressing plasmids. Cell lysates were harvested 72 h posttransfection, and the levels of GFP expression were determined by SDS-polyacrylamide electrophoresis and immunoblot analyses (Figure 2A). In cells that were depleted of Aha1, Cdc37, FKBP4, or p23, expression of the GFP-let7a reporter was significantly increased (Figure 2A).

Gene-silencing efficiency in the cochaperone knockdown cells was calculated by determining the difference in relative GFP expression (normalized to actin) between cells expressing GFP-let7a and GFP-let7XX. The silencing efficiency of pLKO.1-nsc-transfected cells was set as 100%. As shown in Figure 2B, knockdown of these four cochaperones was associated with decreased RNAi activity comparable to knockdown of hAgo2. Consistent with previous reports that Hsp90 is required for loading and activating RISC, these data demonstrate that cochaperones are also required for efficient gene silencing by hAgo2.

To confirm that the observed effects of cochaperone knockdown are not limited to the GFP-based reporter system, we used a secondary RNAi assay based on sensitivity of transfected cells to tumor necrosis factor α and cyclohexamide (Schindler and Foley, 2010). Efficient siRNA-mediated knockdown of caspase-8 increases cell viability in response to these external apoptotic stimuli, and knockdown of Hsp90 cochaperones resulted in less viability, reflecting decreased efficiency of RNAi-mediated silencing (Supplemental Figure S1A). Immunoblot analysis of cell lysates confirmed that hAgo2 and the targeted cochaperones were efficiently knocked down (Supplemental Figure S1B).

Knockdown of Hsp90 cochaperones does not reduce levels of core RNAi components

Inhibition of Hsp90 or its cochaperones can significantly affect the stability of client proteins (Blagosklonny *et al.*, 1995; Bagatell *et al.*, 2001; Wang *et al.*, 2007; Annamalai *et al.*, 2009; Jung *et al.*, 2011), and recent reports described a similar role for Hsp90 in stabilizing unloaded hAgo2 (Johnston *et al.*, 2010; Martinez and Gregory, 2013). Accordingly, it is possible that the decreased RNAi activity associated with cochaperone knockdown was due to reduced stability of core RNAi proteins. To address this potential scenario, we performed immunoblot analyses on lysates of cells knocked down for FKBP4, Cdc37, Aha1, or p23. Levels of Dicer and hAgo2, the core components of the canonical RNAi pathway, were not dramatically affected by depletion of these Hsp90 cochaperones (Figure 3A). Immunoblotting and quantitation of Aha1, Cdc37, FKBP4, and p23 band intensities confirmed that levels of targeted cochaperones were reduced (Figure 3, B and C).

Inability to bind small RNAs prolongs/enhances the association of hAgo2 with Hsp90 and its cochaperones

To better characterize the role for these cochaperones in RISC activation, we sought to identify changes in cochaperone binding that result from mutations affecting the function of hAgo2. Because Hsp90 activity is required for loading small RNAs onto Argonaute proteins (Iki *et al.*, 2010; Iwasaki *et al.*, 2010; Miyoshi *et al.*, 2010), we expected that hAgo2 mutants unable to interact with small RNAs would remain bound to the chaperone complex longer. Conversely, the interactions between Hsp90, its cochaperones, and an endonuclease-dead mutant of hAgo2 that can still load small RNAs but cannot cleave a targeted mRNA should be similar to that for wild-type hAgo2. To test these predictions, we used three previously characterized mutants of hAgo2: PAZ9 and Y529E, which cannot bind small RNAs (Liu *et al.*, 2004; Rudel *et al.*, 2011), and H634P, which lacks endonuclease activity (Liu *et al.*, 2004). We transiently transfected plasmid constructs expressing myc-tagged hAgo2 (wild type, PAZ9, H634P, or Y529E) into HeLa cells for a total of 16 h. Cell lysates were subjected to immunoprecipitation using Sepharose beads conjugated to either anti-myc or an isotype-matched IgG as a control. Input and bound fractions were immunoblotted for Dicer, Hsp90, Hsp70, FKBP4, Cdc37, Aha1, and p23 (Figure 4A).

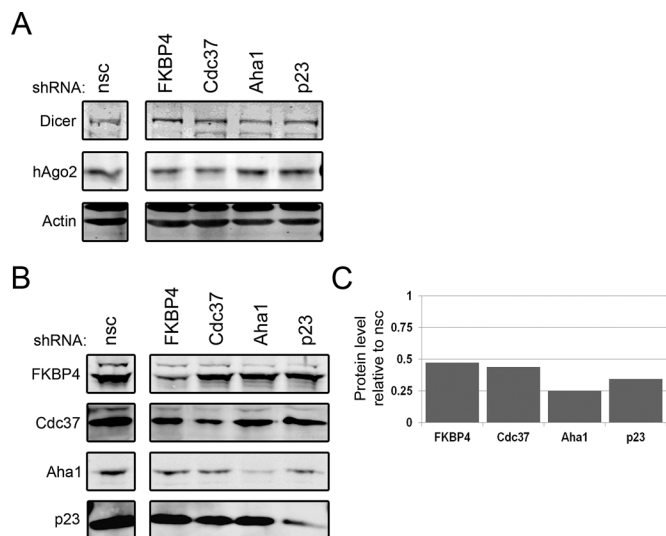


FIGURE 3: Knockdown of Hsp90 cochaperones does not affect the level of RNAi effector proteins. (A) HeLa cells were harvested 72 h after transient cotransfection with pLKO.1 vectors expressing shRNAs against hAgo2, FKBP4, Cdc37, Aha1, p23, or a nonsilencing control (nsc) as a control. Lysates were subjected to SDS-PAGE, and the levels of the RNAi effector proteins Dicer and hAgo2 were determined by immunoblot analysis. (B) Lysates from A were immunoblotted for the targeted cochaperones FKBP4, Cdc37, Aha1, and p23. (C) Signal intensities for FKBP4, Cdc37, Aha1, and p23 were quantitated for each knockdown. The bar graph indicates the average level of expression of the shRNA-targeted protein remaining 72 h posttransfection relative to the nonsilencing control.

Quantitation of the coimmunoprecipitated chaperones/cochaperones revealed a twofold to threefold increase in association of Hsp90, Hsp70, FKBP4, and p23 with the PAZ9 mutant compared with wild-type hAgo2 (Figure 4B). Increased association was also observed between the Y529E mutant and Hsp90 (Supplemental Figure S2, A and B). There was no significant increase in the association between any of the chaperones/cochaperones and myc-hAgo2 H634P (Figure 4B). These results suggest that Hsp90, p23, and FKBP4 bind to unloaded hAgo2 and that, when small RNA binding to hAgo2 is prevented, this complex accumulates to high levels.

DISCUSSION

There are more than 20 cochaperones that regulate multiple aspects of the Hsp90 chaperone cycle (Taipale *et al.*, 2010). Here we further characterize the hAgo2-Hsp90 complex and report that two cochaperones, FKBP4 and p23, coimmunoprecipitate with hAgo2. We also observed that FKBP4 and p23, as well as two cochaperones that do not form a stable complex with hAgo2 (Aha1 and Cdc37), are important for RNAi.

Cdc37 is required for the maturation of many kinases, as it functions to recognize and recruit these but not other clients to Hsp90 (Gerber *et al.*, 1995; Pearl, 2005). Consequently, depletion of Cdc37 results in decreased activity of multiple kinases (Caplan *et al.*, 2007). Although Cdc37 forms stable complexes with Hsp90 and its client kinases (Stepanova *et al.*, 1996; Kimura *et al.*, 1997; Prince *et al.*, 2005), we did not detect it in complex with myc-hAgo2, consistent with the consensus that Cdc37 involvement is restricted to kinase clients of Hsp90. Furthermore, FKBP4 and Cdc37 coimmunoprecipitate in the same complex as a kinase client under buffer conditions similar to those used in the present study (Hartson *et al.*, 2000). Therefore we conclude that the effect of Cdc37 knockdown on RNAi is indirect and

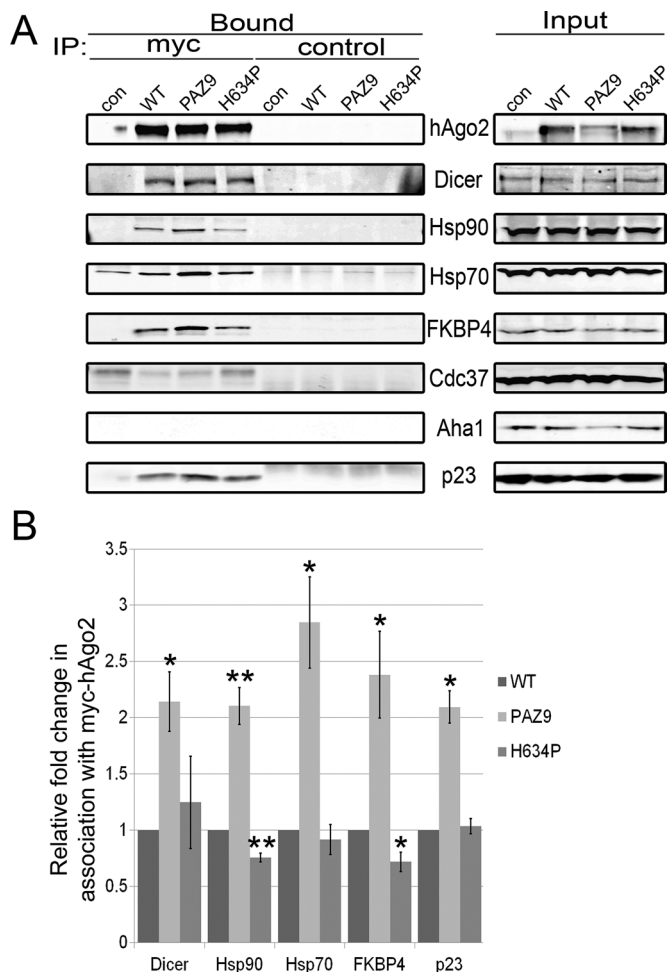


FIGURE 4: Inability to load small RNAs increases association between hAgo2, Hsp90, and cochaperones. (A) HeLa cells transiently transfected with pcDNA3/myc-hAgo2 (WT), pcDNA3/myc-hAgo2-PAZ9 (PAZ9), pcDNA3/myc-hAgo2-H634P (H634P), or empty pcDNA3 (con) were lysed 16 h posttransfection and subjected to immunoprecipitation using Sepharose beads coated with monoclonal anti-myc antibodies (myc) or anti-VSVG (control) as a control. Total cell lysate (Input) and bound fractions were subjected to SDS-PAGE and immunoblotted for hAgo2, Dicer, Hsp90, Hsp70, and cochaperones as indicated. (B) Signal intensities for Dicer, Hsp90, Hsp70, FKBP4, and p23 were quantitated and normalized to level of immunoprecipitated hAgo2. The bar graph indicates the fold change in the amount of each coimmunoprecipitated hAgo2-binding partner analyzed relative to the amount associated with wild-type hAgo2. Error bars indicate SE. * $p < 0.05$; ** $p < 0.01$.

mediated by one or more of its kinase clients. Consistent with this conclusion, hAgo2 is subject to numerous phosphorylation events (Zeng *et al.*, 2008; Rudel *et al.*, 2011), and mutation of these sites have functional consequences for RNAi (Rudel *et al.*, 2011; Horman *et al.*, 2013). Whereas phosphorylation of hAgo2 at Y529 prevents its binding to a small RNA (Rudel *et al.*, 2011) and phosphorylation of S387 inhibits cleavage and increases translational repression (Horman *et al.*, 2013), phosphorylation of other sites within hAgo2 may well increase its activity in RNAi. If so, we expect any kinase(s) responsible for activating hAgo2 would be a client(s) of Hsp90 and Cdc37.

Maturation of client proteins requires energy from the binding and hydrolysis of ATP by Hsp90. The model proposed by Wang *et al.* (2006) predicts that activation of the ATPase activity of Hsp90

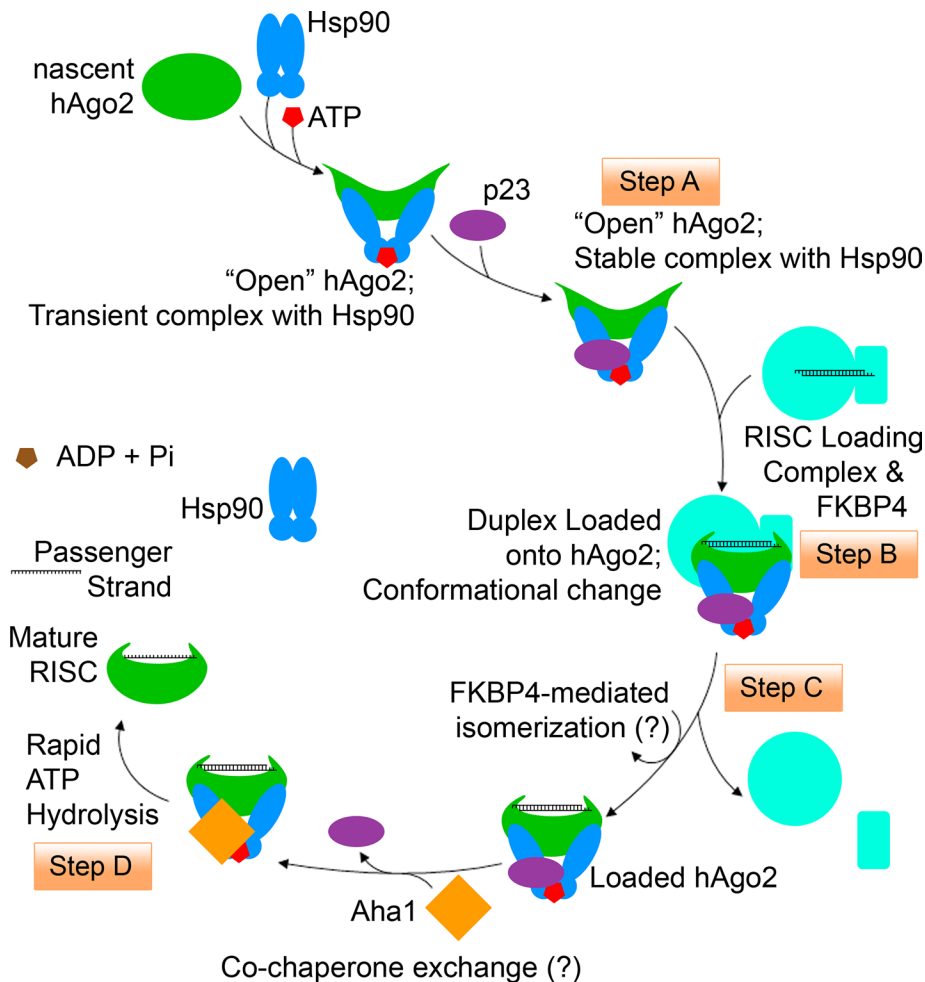


FIGURE 5: Model of cochaperone involvement in Hsp90-mediated maturation of RISC. (A) ATP-binding to Hsp90 facilitates a conformational change in the chaperone–client complex, leading to an “open” conformation of hAgo2. This transient intermediate is stabilized by interaction with p23, which delays the hydrolysis of ATP and release of the client. (B) Interaction with the RISC loading complex and subsequent loading of a small RNA causing a second conformational change in hAgo2. (C) PPLase activity of FKBP4 alters the *cis/trans* bond configuration of hAgo2, stabilizing the loaded conformation of RISC and its interaction with the guide RNA. The isomerization of peptidyl-prolyl bonds in hAgo2 triggers the coordinated exchange of p23 with Aha1. (D) Binding of Aha1 activates the ATPase activity of Hsp90. ATP hydrolysis promotes the rapid resolution of the chaperone–client complex, unwinding and removal of the passenger strand, and release of the mature RISC.

drives resolution of the client–chaperone complex, releasing the mature client. We observed a significant increase in the association between a small RNA-binding mutant of hAgo2 (PAZ9), Hsp90, p23, FKBP4, and Dicer, suggesting that ATPase activation and complex resolution are dependent on successful client maturation. P23 associates with Hsp90 after ATP binding (Richter *et al.*, 2004) and prevents hydrolysis of the nucleoside (Siligardi *et al.*, 2004), thereby stabilizing an otherwise transient conformation of Hsp90. In other words, p23 reduces the kinetics of ATP hydrolysis, which, according to the model of Wang *et al.* (2006), increases “dwell time” and allows for successful client maturation (Dittmar *et al.*, 1997; McLaughlin *et al.*, 2006). The mechanism for prolonged inhibition of ATP hydrolysis by p23 is unclear, but one intriguing possibility is that this cochaperone serves as a molecular sensor for client maturation. In this capacity, p23 could delay ATP hydrolysis and complex resolution until the client has been fully matured (Figure 5A).

of the chaperone complex and releasing the active RISC (Figure 5D). Because ATP hydrolysis by Hsp90 leads to rapid resolution of the chaperone complex, the Hsp90–client–Aha1 complex is likely short lived (Prodromou *et al.*, 2000; Shiau *et al.*, 2006; Koulov *et al.*, 2010). The transient nature of this complex could explain the lack of detectable interaction between Aha1 and hAgo2.

Our data suggest that both FKBP4 and p23 form a stable complex with myc-hAgo2 before its interaction with a small RNA. This appears to conflict with our previous conclusion that the localization of Aha1 and Cdc37 to stress granules reflects a role for them in regulating hAgo2 function (Pare *et al.*, 2009). However, it is important to note that the RISC loading complex components Dicer and TRBP do not localize to P bodies or stress granules (Pare *et al.*, 2009), and it is established that hAgo2 mutants that do not bind small RNAs are not recruited to RNA granules (Leung *et al.*, 2006; Pare *et al.*, 2011). Taken together, these data provide strong evidence that RISC loading occurs in the cytoplasm rather than RNA

A molecular sensor of client maturation necessarily requires a signal from the activated client. We predict that hAgo2 undergoes structural rearrangements before and after loading the miRNA duplex and that the latter triggers progression through the p23-mediated delay in the chaperone cycle (Figure 5B). FKBP4 (also known as FKBP59, FKBP52) is a member of the immunophilin class of cochaperones and has peptidyl-prolyl isomerase (PPLase) activity (Peattie *et al.*, 1992; Smith *et al.*, 1993; Dolinski *et al.*, 1997). Changes to the *cis/trans* bond configuration of proline residues within hAgo2 may be necessary for it to stably associate with small RNAs and could be integral to the conformational change proposed earlier (Figure 5C). FKBP4 forms a complex with hAgo2, Hsp90, p23, and Dicer that builds up when small RNA loading to hAgo2 is prevented (e.g., PAZ9 mutant). In keeping with this observation, it was reported that AGO1 of *Arabidopsis thaliana* associates with the PPLases Cyp40, FKBP65, and FKBP62, the plant orthologue of FKBP4 (Iki *et al.*, 2010). A ligand-induced conformational change in the client that is detected by cochaperones of Hsp90 and promotes the release of the mature client would be a novel means of regulating the chaperone cycle and warrants continued investigation.

Aha1 binds Hsp90 in a coordinated exchange with p23 (Harst *et al.*, 2005) and stimulates the weak ATPase activity of the chaperone (Obermann *et al.*, 1998; Panaretou *et al.*, 1998; Panaretou *et al.*, 2002). ATP hydrolysis is believed to promote resolution of the client–chaperone complex (Hessling *et al.*, 2009). In the present study, we demonstrate that knockdown of Aha1 decreases the efficiency of RNAi. We hypothesize that Aha1 stimulates the ATPase activity of Hsp90 after a conformational change in hAgo2 associated with small RNA binding, driving the resolution

granules and that this process is likely facilitated by Hsp90, FKBP4, and p23. Moreover, p23 and FKBP4 are required for the maturation of steroid hormone receptors (Dittmar *et al.*, 1997; Morishima *et al.*, 2003; Riggs *et al.*, 2003; Pratt *et al.*, 2004; Wochnik *et al.*, 2005). Therefore it is tempting to speculate that RISC is loaded in a manner analogous to these receptors and that recruitment of Hsp90, Hsp70, HOP, p23, Cdc37, and Aha1 to stress granules is unrelated to this process. Of importance, these conclusions are not mutually exclusive of the possibility that an Hsp90 chaperone complex is required for regulating a separate, as-yet-unknown aspect of hAgo2 function, downstream of loading, during conditions of cellular stress.

In summary, we identified a subset of Hsp90 cochaperones involved in activating hAgo2 and discussed their previously described regulatory roles in the context of Hsp90-mediated maturation of hAgo2. There is a shortage of clients that can be used to study the regulation and progression of the Hsp90 chaperone cycle. We predict that hAgo2 maturation is analogous to maturation of steroid hormone receptors. Further, because of its soluble ligand, easily assayed activity, and tractable nature (nascent, unloaded; bound to a small RNA duplex; bound to the guide strand only; in complex with target mRNA), it may serve as an excellent tool with which to further study the mechanism of Hsp90 chaperone complexes.

MATERIALS AND METHODS

Antibodies

The mouse monoclonals anti-myc (9E10) and anti-VSV-G (P5D4) were prepared from hybridoma cell lines in the Hobman laboratory. The rabbit polyclonal antibody (2D4) was generated against the PAZ domain of hAgo2. The rabbit polyclonal anti-Aha1 was described previously (Wang *et al.*, 2006). Other primary antibodies were from the following sources: rabbit polyclonal anti-GFP from L. Berthiaume (University of Alberta, Edmonton, Canada); mouse monoclonals anti-Dicer (ab14601), anti-hAgo2 (ab57113), anti-FKBP4 (ab59460), and anti-p23 (ab2814), and rabbit polyclonal anti-FKBP4 (ab97306) from Abcam (Cambridge, MA); goat polyclonal anti-HSP90 (sc-1055) from Santa Cruz Biotechnology (Santa Cruz, CA); mouse monoclonal anti-Hsp70 (SPA-810) from Enzo Life Sciences (Farmingdale, NY); mouse monoclonals anti-Cdc37 (MA3-029) and anti-p23 (MA3-414) from Thermo Fisher Scientific (Waltham, MA). Goat anti-rabbit conjugated to Alexa 750 (A21039), donkey anti-mouse conjugated to Alexa 680 (A10038), and donkey anti-goat conjugated to Alexa 680 (A21084) were purchased from Life Technologies (Carlsbad, CA). Goat anti-human conjugated to horseradish peroxidase was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Plasmids and siRNAs

The plasmids pcDNA3/mycHis-hAgo2-PAZ9 and pcDNA3/mycHis-hAgo2-H634P were gifts from G. Hannon (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). pcDNA3/mycHis-hAgo2-WT was generated using pcDNA3/mycHis-hAgo2-H634P as a template in a site-directed mutagenesis reaction using QuikChange Site-Directed Mutagenesis kit (Agilent, Santa Clara, CA) and the primers hAgo2-H634PtoWT-for (5'-GCG CGT GCA GCA GCA CCG GCA GGA GAT CAT ACA AGA C) and hAgo2-H634PtoWT-rev (5'-GTC TTG TAT GAT CTC CTG CCG GTG CTG CTG CAC GCG C). pcDNA3/mycHis-Y529E was also generated by site-directed mutagenesis as described, using pcDNA3/mycHis-hAgo2-WT as the template and the primers hAgo2-WTtoY529E-for (5'-ATC CTG CCC GGC AAG ACG CCC GTG GAA GCC GAG GTC AAA CGC GTG GGA GAC ACG GTG CTG GGG) and hAgo2-WTtoY529E-rev (5'-CCC CAG CAC CGT GTC TCC CAC GCG TTT GAC CTC GGC TTC CAC GGG CGT

CTT GCC GGG CAG GAT). pLKO.1 plasmids encoding shRNAs against Ago2, FKBP4, Cdc37, Aha1, p23, and the nonmammalian shRNA control were purchased from Sigma-Aldrich (St. Louis, MO). pEGFP-N1 was purchased from Clontech (Mountain View, CA), and pEGFP-N1/let7a was a gift from E. Jan (University of British Columbia, Vancouver, Canada). pEGFP-N1/let7XX was constructed by ligating the duplex (5'-AACTGAGGTTTCGTTGTATAGTC; 5'-AATTGAACTATACAACGAACTACTCAGTT) into pEGFP-N1/let7a digested with *MfeI* and *HpaI*.

SMARTpool siRNAs against hAgo2 (27161), FKBP4 (2288), Cdc37 (11140), Aha1/Ahsa1 (10598), and p23/PTGES3 (10728) were from Thermo Fisher Scientific, and the nonsilencing control (AM4611) was from Life Technologies. The two oligonucleotides (5'-GAUACUGUCUGAUCAUCAAUUCAAA and 5'-UUUGAAUUGAUGAUCAGACAGUAUCCC), when annealed, formed the Dicer-substrate siRNA specific for caspase-8 and were purchased from Integrated DNA Technologies (Coralville, IA).

Cell culture and transfection

Puromycin (P8833), Alamar Blue/resazurin sodium salt (R7017), and cyclohexamide (C7698) were from Sigma-Aldrich, and tumor necrosis factor α (11 371 843 001) was from Roche (Penzberg, Germany). All other cell culture and transfection reagents were purchased from Life Technologies. HeLa cells (ATCC, Manassas, VA) were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, at 37°C and 5% CO₂. DNA transfections were performed using Lipofectamine 2000 in OptiMEM, and siRNA transfections were performed in Lipofectamine RNAiMAX, both following manufacturer's recommended protocol. To enrich for cells harboring pLKO.1 plasmids, medium was replaced 24 h after transfection with growth medium containing 1 μ g/ml puromycin for 48 h.

Immunoblotting

Total cell lysates were lysed in 1 \times Passive Lysis Buffer (Promega, Madison, WI) and assayed for total protein concentration using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Samples were subsequently prepared for electrophoresis by adding 5 \times Laemmli gel loading buffer supplemented with 5% β -mercaptoethanol. Samples were then boiled and resolved in SDS-polyacrylamide gels, transferred to 0.45 μ m nitrocellulose membrane (162-0115; Bio-Rad), and incubated with primary antibodies diluted in 5% skim milk powder in phosphate-buffered saline (PBS)/Tween 20. After washing, samples were incubated with secondary antibodies conjugated to Alexa 680, Alexa 750, or horseradish peroxidase. The membranes were scanned with an Odyssey infrared imaging system (model 9120) and software (Li-Cor Biosciences, Lincoln, NE) or developed using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific), imaged using FluorChem Q and image acquisition software (Cell Biosciences, Santa Clara, CA), and analyzed with ImageJ software (National Institutes of Health, Bethesda, MD).

Primary antibody cross-linking

Monoclonal antibodies 9E10 and P5D4 were mixed with protein G-Sepharose 4 fast flow beads (17-0618-01; GE Healthcare, Little Chalfont, United Kingdom) 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 dimethylpimelidate 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.

Immunoprecipitation of Argonaute2 complexes

Cells were lysed in 50 mM Tris, pH 7.2, 20 mM NaCl, 1 mM MgCl₂, and 1% Triton X-100 supplemented with Complete protease inhibitor to 0.5× (Roche, Indianapolis, IN). Cell lysates were cleared by centrifugation at 15,000 × g for 10 min. Benzamide was from EMD Millipore (Billerica, MA). For immunoprecipitation of endogenous proteins, 10 µg of monoclonal antibody was added to clarified lysate, rotated for 2 h at 4°C, and then incubated with protein G-Sepharose blocked with 2% bovine serum albumin for 30 min (Sigma-Aldrich). Myc-tagged Ago2 complexes were immunoprecipitated with cross-linked protein G beads for 1 h at 4°C. All immunoprecipitations were washed once with binding buffer after incubation, and then beads were boiled in sample buffer and analyzed by SDS-PAGE and immunoblotting.

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