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Characterization of the interaction of Aha1 with components of the Hsp90 chaperone machine and client proteins

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ABSTRACT

The activator of Hsp90 ATPase, Aha1, is an Hsp90 co-chaperone that has been suggested to act as a general stimulator of Hsp90 function. In this report, we have characterized the interaction of Aha1 with Hsp90 and its co-chaperones in rabbit reticulocyte lysate (RRL) and in HeLa cell extracts. Complexes formed by Aha1 with Hsp90 in RRL were stabilized by molybdate and contained the co-chaperones FKBP52 and p23/Sba1, but lacked HOP/Sti1 and Cdc37. Aha1 complexes isolated from HeLa cell extracts also contained Hsp70 and DNAJA1. Over-expression of Aha1 has been reported to stimulate the activity of v-Src and steroid hormone receptors ectopically expressed in yeast, however, no interaction between Aha1 and nascent v-Src or the progesterone receptor could be detected in RRL. Contrary to expectations, over-expression of Aha1 also inhibited the rate of Hsp90-dependent refolding of denatured luciferase. A number of potential client proteins that specifically associated with Aha1 were identified by liquid chromatography/ tandem mass spectrometry (LC–MS/MS) and verified by Western blotting. The proteins identified suggest that Aha1 may play roles in modulating RNA splicing and DNA repair, in addition to other cellular processes.

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1. Introduction

Hsp90 is a molecular chaperone that is required for the proper function of numerous proteins in eukaryotic cells (reviewed in [1–3]). Hsp90 functions in conjunction with a number of partner proteins commonly referred to as co-chaperones [1–3]. The Hsp90 co-chaperone Aha1 was so named because it was found to be an activator of Hsp90's ATPase activity [4,5]. Hsp90 and Aha1 interact physically [4–8], with Aha1's N-terminal domain binding primarily to Hsp90's middle and its C-terminal domain interacting with Hsp90's N-terminal domain [4,7,9,10]. High affinity binding and potent stimulation of Hsp90's ATPase activity have been found to require both of Aha1's domains [4,5].

While Aha1 and its homolog Hch1p are not required for viability in yeast, studies have demonstrated that the genes become crucial for cell viability under non-optimal growth conditions when Hsp90 levels are limiting [4,5,11]. In addition, in yeast strains where Hsp90 activity was limiting, over-expression of Hch1p enhanced the maturation of a heterologous Hsp90 target protein, v-Src [11], while deletion of the genes coding for Hch1p and Aha1 markedly decreased the activity of ectopically expressed v-Src [4,5]. Furthermore, studies indicate that Aha1 over-expression also enhanced the activity of ectopically expressed steroid hormone receptors (SHR) in yeast [12]. These findings, together with the observation that Aha1 expression is stress-inducible have led to the current hypothesis that Aha1 is a co-chaperone that generally enhances Hsp90 function through its ability to stimulate Hsp90's ATPase activity [5].

Implicit in this hypothesis is the posit that Aha1 is a general co-chaperone for Hsp90-dependent clients. However, to date there are only a limited number of reports documenting complexes of Hsp90 and Aha1 containing bound client, or the effect of Aha1 on client protein activity. Aha1 was originally described as a 38-kDa protein (p38) that interacted with VSV-G and was postulated to associate transiently with endoplasmic reticulum (ER) membranes, possibly modulating the transit of VSV-G and other cargo proteins from the ER to the Golgi [13]. Consistent with this notion, Aha1 [9,14,15] and Hsp90 [16,17] have been shown to interact with the cystic fibrosis transmembrane conductance regulator (CFTR) and its folding mutant CFTR(ΔF508), in addition to wild-type and mutant melanocortin-4 receptors (MC4R, [18]), and modulate their transport to the plasma membrane. Mutations that impair Aha1 function [9], down regulation of Aha1 expression [15], or displacement of Aha1 from CFTR(ΔF508) [14] rescue misfolding of CFTR(ΔF508) and stimulate its maturation and transport to the plasma membrane. Aha1 also interacts with Hsp90 complexes containing bound adenine nucleotide transporter (ANT1), a mitochondrial pre-protein that is targeted by cytoplasmic chaperone transport complexes to the outer mitochondrial membrane [19]. In addition, Aha1 has been reported to interact with the SR-protein specific kinase (SRPK1, [20]), C-RAF and Akt [21], and GTP

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cyclohydrolase I [22]. Recently, Aha1 was also reported to be a negative regulator of MAL-activator activity in yeast [23].

Herein, the interaction of Aha1 with Hsp90 and its co-chaperones was characterized in rabbit reticulocyte lysate (RRL) and HeLa cell extracts, as a number of ambiguous observations have been previously reported in the literature [5,8,12]. Furthermore, we have tested the hypothesis that the Aha1-induced increase in v-Src [4,5,11,12], Akt [21] and SHR activity [12] that has been previously reported, was due to a direct interaction of Aha1 with the Hsp90-complexes containing bound nascent client. We report that Aha1 could not be detected in complexes formed between Hsp90 and nascent v-Src, Akt or the progesterone receptor (PR), even when the binding of Hsp90 to v-Src, Akt or PR was stabilized by the addition of molybdate: a condition that also stabilizes the binding of Aha1 to Hsp90. Furthermore, over-expression of Aha1 did not enhance v-Src activity, increase phosphotyrosine levels in HeLa cells or stimulate the rate of Hsp90-dependent refolding of denatured luciferase in RRL [24]. In addition, a number of potential client proteins that were co-adsorbed with Aha1 from HeLa cell extracts were identified by LC–MS/MS and verified by Western blotting.

2. Materials and methods

2.1. Analysis of the interactions of Aha1 with Hsp90, co-chaperones and potential client targets

His-tagged Aha1 was synthesized and radiolabeled via coupled transcription/translation (TnT) in RRL for 40 min at 30 °C as previously described [25,26]. His-tagged Aha1 was directly immuno-absorbed with anti-IgG-agarose containing bound anti-(His$_5$) antibody (Qiagen) [25,26]. Hsp90 (OSU anti-Hsp90, [27]), Hsp70 (BB70 monoclonal, [28]), Cdc37 (anti-Cdc37 polyclonal, [29]), p23 (JJ5 monoclonal, [30]), FKBP52 (EC1 monoclonal, [31]), Hip (2G6 monoclonal, [32]) or Hop (F5 monoclonal, [28]) were adsorbed with polyclonal or monoclonal antibodies directed against each of the proteins. Immunoprecipitates were washed once with 10 mM PIPES (pH 7.2), 100 mM NaCl and 0.5% Tween-20 [e.g., 10 mM PIPES buffer (pH 7.2) containing 100 mM NaCl and 0.5% Tween-20 = P100T] and 3 times with P50T and co-adsorbing proteins were analyzed by SDS-PAGE and Western blotting as previously described [25,26].

v-Src or PR was synthesized by TnT in RRL in the presence or absence of 15 μg/ml geldanamycin (GA) at 30 °C for 30 min [25,26]. To examine kinase-binding or steroid hormone receptor binding activity of Aha1, RRL containing radiolabeled v-Src or progesterone receptor (PR) was mixed with RRL containing [^{35}S]Aha1, synthesized as described above, followed by an additional 30 min incubation at 30 °C. His-tagged Aha1 or Hsp90 was adsorbed with anti-His-tag or anti-Hsp90 antibody in the presence or absence of 20 mM molybdate. Unless otherwise indicated in the figure legends, immunoresins were washed 4 times with P50T in the presence or absence of 20 mM molybdate as indicated in the legends of the figures. To assess the interaction of endogenous Aha1 with Akt, Flag-tagged Akt was generated by TnT and immunoadsorbed with anti-Flag antibody as described above. Samples were analyzed by SDS-PAGE, Western blotting and/or autoradiography.

2.2. In vitro v-Src protein kinase assay

[^{35}S]-Labeled v-Src was synthesized by TnT in RRL in the presence or absence of 15 μg/ml GA for 30 min at 30 °C. The lysate was then mixed with TnT lysate containing newly synthesized [^{35}S]-labeled Aha1 or mock lysate followed by another 30 min incubation at 30 °C. The mixtures were immunoprecipitated with anti-Src monoclonal antibody for 1 h at 4 °C with gentle rocking. The immunoresins were then washed twice with ice-cold P500T and twice with P150. Similar results were obtained when immunoresins were washed twice with

P300 and twice with P100. The v-Src kinase assay was carried out by incubating the immunoresins with 0.5 μg/ml acid-treated enolase and 1.0 mM ATP in kinase buffer containing 10 mM PIPES pH 7.4, 150 mM NaCl, and 20 mM MnCl$_2$ for 5 min at 37 °C. Boiling SDS sample buffer was then added into the assay mixtures to end the reaction, and samples were analyzed by SDS-PAGE on an 8% gel and then transferred to PVDF membrane. The newly synthesized v-Src and His$_6$-Aha1 were detected by autoradiography. The co-adsorbed Hsp90 and phosphotyrosine were detected by Western blotting with anti-Hsp90 antibody [27] and anti-phospho-tyrosine monoclonal antibody (P-Tyo-100, Cell Signaling, Cat #9411), respectively. The endogenous and His-tagged Aha1 in the input was detected by Western blotting with rabbit polyclonal anti-Aha1 [13].

2.3. Akt kinase assay

Human Aha1 sequence was cloned into pcDNA3.1 vector with an upstream Flag-epitope tag. Plasmid encoding Flag-Aha1was trans-fected into HeLa cells cultured on a 6-well plate for 48 h. Cells were washed once with 20 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl (phosphate buffered saline, PBS) and then incubated with ice-cold 1× cell lysis buffer (Akt Kinase Assay Kit, Cell Signaling, catalog #9840) for 5 min. Cells were scraped off the plate and sonicated on ice. Cell lysates were centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatants were incubated with immobilized phospho-Akt (Ser473) rabbit antibody beads with gentle rocking for 4 h at 4 °C followed by washing with 1× cell lysis buffer and then 1× kinase buffer. Immuno-beads were suspended in 1× kinase buffer with 0.2 mM ATP and 0.2 μg GSK-3 fusion protein and then incubated for 30 min at 30 °C. Kinase assay reactions were terminated by adding SDS sample buffer, followed by boiling for 5 min. After SDS-PAGE and electro-transfer, PVDF membranes were Western-blotted with anti-phospho-GSK-3α/β (Ser21/9) rabbit monoclonal antibody (Akt Kinase Assay Kit), polyclonal mouse anti-Hsp90 [27], polyclonal rabbit anti-Aha1 [13], anti-Akt1/2/3 (H-136) (Santa Cruz, catalog #sc-8312), and anti-phospho-Akt (Ser473) (Cell signaling, catalog #9271S).

2.4. Luciferase renaturation assay

Refolding of firefly luciferase in RRL was carried out as previously described with minor modifications [24]. Luciferase (0.5 mg/ml, Sigma) was dissolved in stability buffer (25 mM Tricine–HCl (pH 7.8), 8 mM MgSO$_4$, 0.1 mM EDTA, 10 mg/ml bovine serum albumin, 10% glycerol, and 1% Triton X-100), and denatured by heating at 40 °C for 10 min. His-tagged Aha1 was synthesized by TnT in RRL for 30 min at 30 °C. Naïve TnT lysate containing no plasmid was similarly incubated and used as a control. The refolding reaction was initiated by the addition of 1 μl denatured luciferase to the TnT lysate. The luciferase activity present in the TnT lysates was measured at 0 min and after a 10 min incubation at 30 °C, by the addition of a 2 μl aliquot of the TnT lysate into 50 μl of assay buffer (25 mM Tricine–HCl (pH 7.8), 8 mM MgSO$_4$, 0.1 mM EDTA, 33 μM DTT, 470 μM D-luciferin, 240 μM coenzyme A, and 0.5 mM ATP) followed by a 10 s integration time to measure relative light unit (RLU) production using a Lumac Bioluminometer (3 M). Reactions were carried out in triplicate and the experiment was repeated at least three times.

2.5. Identification of Aha1-interacting proteins by LC–MS/MS and Western blotting

HeLa cells were transfected with Flag-Aha1 plasmid or empty vector using Lipofectamine 2000 transfection reagent (Invitrogen, catalog #11668019) and incubated at 37 °C in a humidified incubator with 5% CO$_2$ for 48 h. Cells were dissociated from tissue culture flasks (Cell Dissociation Solution Non-enzymatic 1×, Sigma, catalog

#C5789) and washed once with PBS before lysing the cells in RIPA lysis buffer containing 50 mM HEPES (pH 7.4), 2% Igepal CA-630, 0.1% SDS, 150 mM NaCl, 0.75% deoxycholic acid, 1 mM EGTA, 1 mM NaF, 2 mM Na_3VO_4, PhosSTOP phosphatase inhibitor cocktail (Roche, Catalog #04906837001), complete mini protease inhibitor cocktail (Roche, Catalog #04693124001) and 20 mM sodium molybdate. Cell lysates were then sonicated, shaken at 4 °C for 20 min and centrifuged at 14,000 rpm for 10 min. The supernatants were mixed with anti-Flag agarose (Sigma, catalog #A2220) for 2 h at 4 °C. The anti-Flag resins were then washed three times with P50T containing 20 mM molybdate and once with P20T containing 20 mM molybdate, followed by elution of the immunoadsorbed proteins by boiling in SDS-sample buffer and separation of the samples by SDS-PAGE on 8% gels. For Western blot analysis of co-adsorbed proteins and co-chaperones, gels were transferred to PDVF membranes. For LC–MS/MS identification of co-adsorbed proteins, gels were stained with colloidal-Coomassie blue G250 and destained, after which protein bands were excised, and destained by extensive washing with 50% acetonitrile/50 mM ammonium bicarbonate pH 8, dehydrated with 100% acetonitrile, and dried briefly. Dried acrylamide pieces were rehydrated with 10 mM tris-2-carboxyethyl-phosphine (TCEP), 50 mM ammonium bicarbonate, and reduced for 1 h at room temperature. Subsequently, the reducing buffer was replaced with 55 mM iodoacetamide in 50 mM ammonium bicarbonate, and samples were alkylated for 1 h at room temperature in the dark. Samples were then rinsed with ammonium bicarbonate, dehydrated with acetonitrile, and rehydrated/infiltrated with trypsin solution containing 8 μg/ml of trypsin in 50 mM ammonium bicarbonate. After overnight digestion at 37 °C, the trypsinolytic peptide products were extracted with 0.5% trifluoroacetic acid, and used for subsequent analysis by mass spectrometry.

Samples were analyzed on a hybrid LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) coupled to a New Objectives PV-550 nanoelectrospray ion source and an Eksigent NanoLC-2D chromatography system. Peptides were analyzed by trapping on a 2.5 cm ProteoPrepII pre-column (New Objective) and analytical separation on a 75 μm ID fused silica column packed in house with 10-cm of Magic C18 AQ, terminated with an integral fused silica emitter pulled in house. Peptides were eluted using a 5–40% acetonitrile/0.1% formic acid gradient performed over 40 min at a flow rate of 300 nl/min.

During each one-second full-range FT-MS scan (nominal resolution of 60,000 FWHM, 300 to 2000 m/z), the three most intense ions were analyzed via MS/MS in the linear ion trap. MS/MS settings used a trigger threshold of 1000 counts, monoisotopic precursor selection (MIPS), and rejection of parent ions that had unassigned charge states, were previously identified as contaminants on blank gradient runs, or were previously selected for MS/MS (data dependent acquisition using a dynamic exclusion for 150% of the observed chromatographic peak width). Column performance was monitored using trypsin autolysis fragments (m/z 421.76), and via blank injections between samples to assay for contamination.

2.6. Data analysis

Centroided ion masses were extracted using the extract_msn.exe utility from Bioworks 3.3.1 and were used for database searching with Mascot v2.2.04 (Matrix Science) and X! Tandem v2007.01.01.1 (www.thegpm.org). Searches were conducted in IPI_human_022209 database using the following search parameters: parent ion mass tolerance 10 ppm; fragment ion tolerance 0.8 Da; one missed tryptic cleavage; variable modifications of pyroglutamate cyclization of N-terminal Gln, oxidation of Met, formylation or acetylation of the protein N-terminus, and carbamidomethyl cysteine. Peptide and protein identifications were validated using Scaffold v2.2.00 (Proteome Software) and the PeptideProphet algorithm [33]. Probability thresholds were greater than 99.0% probability for protein

identifications, based upon at least 2 unique peptides identified with 80.0% certainty. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

The interaction of Aha1 with its putative binding proteins identified by MS analysis was further examined by Western blotting of immuno-adsorbed samples prepared from HeLa cells transfected with Flag-tagged Aha1 as described above, after SDS-PAGE and electrotransfer to PVDF membrane. Each membrane was incubated with individual primary antibody, anti-Hsp70 (StressMarq, catalog #SMC-104A/B, mouse monoclonal), anti-DNAJA1 (Thermo, catalog #MS-225, HDJ-2 mouse monoclonal), anti-RACK1 (Santa Cruz, catalog #sc-17754), anti-PPM1G (Abcam, catalog #ab70794), anti-IQGAP1 (Santa Cruz, catalog #sc-10792), anti-Topoisomerase II alpha (Abcam, catalog #ab45175), anti-SRPK1 (BD Biosciences, catalog #611072), anti-DNA-PKcs (Abcam, catalog #ab1832), anti-PKN2 (Abcam, catalog #ab32395), and anti-Ku70/Ku80 (Abcam, catalog #ab53126) with gentle rocking overnight at 4 °C. After incubation with alkaline phosphatase-conjugated secondary antibody for 2 h at room temperature, protein binding was detected by NBT (nitro-blue tetrazolium chloride)/BCIP (5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt) reaction or ECL detection kit (Tropix CDP Star Ready-to-Use with Emerald II™, part #T2216).

3. Results

3.1. Effect of Hsp90 inhibitors on its interaction with Aha1

The binding of Hsp90 to client proteins and its interactions with co-chaperones are modulated by the binding and hydrolysis of ATP, which drives Hsp90 through its reaction cycle (reviewed in [1–3,34]). Geldanamycin, molybdate and novobiocin inhibit Hsp90 function by interrupting Hsp90's progression through its reaction cycle, with each compound having distinct effects on Hsp90's conformation and its interactions with client proteins and co-chaperones [1–3,34]. Thus, we examined the interaction of Aha1 with Hsp90 in the presence or absence of geldanamycin, molybdate and novobiocin to determine whether Aha1 preferentially interacted with a specific conformation of Hsp90. In addition, since the interaction of client proteins with Hsp90 also stimulates Hsp90's ATPase activity [35], we wished to address the question of whether Aha1's interaction with Hsp90 had the characteristics of a co-chaperone or an Hsp90 client.

His-tagged Aha1 was generated by TnT in RRL and immunoadsorbed in the presence or absence of molybdate. In the presence of buffer containing low salt, a very weak interaction between Aha1 and Hsp90 was detected by Western blotting, that was markedly stabilized by the presence of molybdate (Fig. 1A). Since molybdate characteristically stabilizes Hsp90–client complexes to washes with buffer containing high salt [36–38], while Hsp90–co-chaperone interactions are salt labile, we examined the salt stability of Hsp90–Aha1 complexes (Fig. 1A). The amount of Hsp90 that co-adsorbed with Aha1 was reduced to undetectable levels upon washing with buffer containing 0.5 M NaCl (Fig. 1A). Thus, the instability of Hsp90–Aha1 complexes in the presence of high NaCl concentrations is consistent with it interacting with Hsp90 as a co-chaperone rather than a client.

The effect of geldanamycin and novobiocin on Hsp90–Aha1 complex formation was then examined (Fig. 1B and C, respectively). We have previously shown that pre-incubation of lysate with geldanamycin blocks the ability of molybdate to alter Hsp90's conformation and stabilize "late" Hsp90 complexes containing bound client [36]. Hsp90 was immunoadsorbed from TnT lysate that was or was not pre-incubated with geldanamycin (Fig. 1B). A weak interaction between [³⁵S]Aha1 and Hsp90 was detected in the absence of molybdate. Again, the addition of molybdate stabilizes the interaction of [³⁵S]Aha1 with Hsp90. However, pre-incubation of
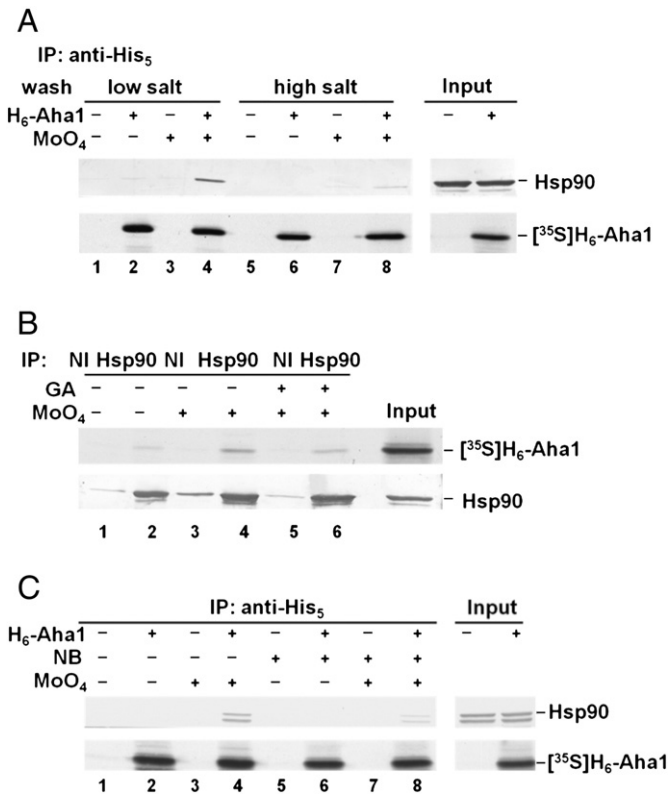
A

IP: anti-His5

B

C

Fig. 1. The effect of Hsp90 inhibitors and salt concentration on the interaction of Aha1 with Hsp90. (A) [^{35}S]-labeled His-tagged Aha1 (+) was synthesized by TnT in reticulocyte lysate at 30 °C for 30 min followed by the addition (or not) of 15 mM molybdate (MoO$_4$) at the end of the incubation. The newly-synthesized His-tagged Aha1 was immunoprecipitated (IP) with anti-His antibody resin. Resins were subsequently washed with buffer containing low salt (P50T) or high salt (P500T), and co-adsorbing Hsp90 and [^{35}S]-Aha1 were detected by Western blotting and autoradiography as described under Section 2.1. Naïve RRL containing no plasmid (−) was used as the control for non-specific binding. (B) [^{35}S]-His-tagged Aha1 was synthesized by TnT in RRL at 30 °C for 30 min following the addition of 15 μg/ml GA (lane 5, 6) or DMSO (vehicle control, lanes 1, 2, 3, 4). After 20 min of further incubation, the mixtures were supplemented with 15 mM molybdate (lanes 3, 4, 5, 6) or vehicle control water (lanes 1, 2) and samples were immunoprecipitated with anti-Hsp90 antibody resin, washed with P50T and analyzed for co-adsorbed Aha1 by SDS-PAGE and autoradiography. (C) [^{35}S]-His-tagged Aha1 was synthesized in TnT reaction for 30 min followed by the addition of 15 mM molybdate (lanes 3, 4, 7, 8) and/or 5 mM novobiocin (lanes 5, 6, 7, 8). After an additional 20 min of incubation, the samples were immunoprecipitated with anti-His antibody resin, washed with P50T and analyzed for co-adsorbed Hsp90 by Western blotting, as described under Section 2.1. NI: control non-immune IgG.

the lysate with geldanamycin prior to the addition of molybdate reduced Aha1 binding to levels near that observed with no additions (Fig. 2A). Thus, geldanamycin blocked molybdate's ability to stabilize the binding of Aha1 to Hsp90.

Novobiocin, on the other hand, has dominant effects to molybdate, stimulating client release from Hsp90-kinase complexes pre-formed in the presence of molybdate, and disrupting the interaction between Hsp90 and co-chaperone components common to "late" chaperone complexes [26]. Addition of novobiocin, caused a marked reduction in the molybdate-stabilized interaction of Hsp90 with Aha1 (Fig. 1C), suggesting that Aha1 did not interact preferentially with novobiocin-bound Hsp90. Overall, the data suggest that Aha1 preferentially interacts with the molybdate-bound conformation of Hsp90.

3.2. Presence of other Hsp90 co-chaperone partners in Hsp90–Aha1 complexes

Several reports have examined the interaction of Aha1 with Hsp90 and a number of its co-chaperone partners in vitro using purified

recombinant proteins [5,8,12]. While the reports agree that Aha1 can be present in Hsp90 complexes containing Cyp40, contradictory results were reported on whether Aha1 could associate with Hsp90 complexes containing bound HOP/Sti1p, Cdc37 or p23/Sba1 [5,8,12]. However, the reports did agree that Aha1 directly associated with Hsp90, as Aha1 did not interact directly with any of Hsp90's other co-chaperone partners [5,8,12].

Thus, we examined the ability of antibodies directed against Hsp90, Hsp70, Hip, p60/Hop, Cdc37, FKBP52 and p23 to co-immunoadsorb [^{35}S]-His-tagged Aha1 to determine the Hsp90/co-chaperone complexes with which Aha1 interacted in the RRL system (Fig. 2A and B). Aha1 was specifically co-adsorbed with Hsp90, FKBP52 and p23. No Aha1 was co-immunoadsorbed with the Hsp90 co-chaperones Cdc37, Hip or HOP above the level present in the controls for non-specific binding. Immunoadsorption of Hsp70 pulled down an amount of [^{35}S] Aha1 that was slightly above background (Fig. 2A). However, the results with the anti-Hsp70, Hip, and Cdc37 pull downs were somewhat equivocal, as significantly less Hsp90 was co-adsorbed compared to the anti-Hsp90, -FKBP52, -p23 and -HOP pull downs.

Subsequently, the interaction of Aha1 with Hsp90 and its co-chaperones was investigated in HeLa cells that were transfected to over-express Flag-tagged Aha1. Hsp90 (not shown) and p23 were detected to co-adsorb with Aha1 from lysates of HeLa cells by Western
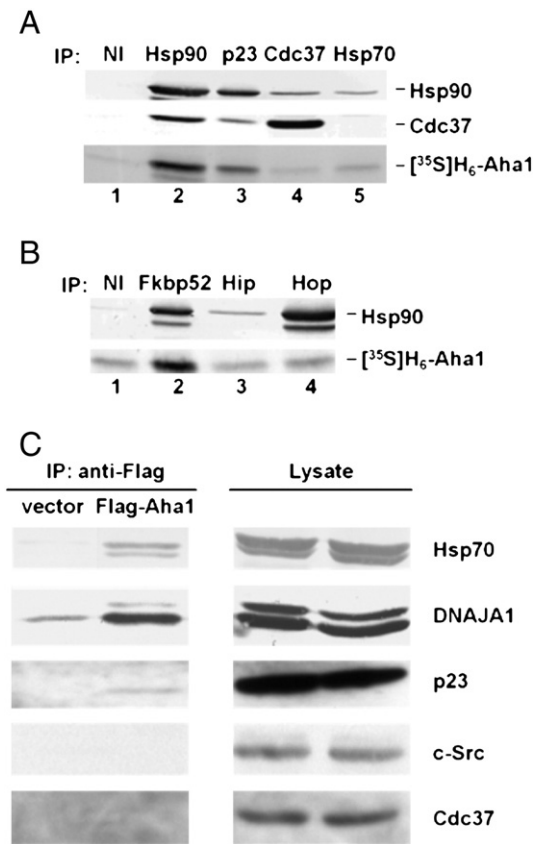
A

B

C

Fig. 2. Interaction of Aha1 with Hsp90 co-chaperones in RRL and Hela cell extracts. [^{35}S]-His-tagged Aha1 was translated in TnT reactions at 30 °C for 30 min. Components of the Hsp90 machine were immunoprecipitated (IP) with (A) anti-Hsp90, anti-p23, anti-Cdc37, or anti-Hsp70, and (B) anti-FKBP52, anti-HIP, or anti-HOP resin in the presence of 15 mM molybdate. Hsp90 and Cdc37 were detected by Western blotting, and [^{35}S]-His-tagged Aha1 binding was detected by autoradiography. NI: control non-immune IgG. (C) Association of Aha1 with Hsp90 co-chaperones in HeLa cells. Flag-Aha1 was expressed in HeLa cells and immunoprecipitated from cell extracts as described under Section 2.5. Samples were analyzed by SDS-PAGE and Western blotting for the presence of co-adsorbing Hsp70, DNAJA1, p23, c-Src, and Cdc37. Lysate: input. HeLa cells transfected with empty vector were used as the control for no-specific binding.

blotting above the background level of nonspecific binding (Fig. 2C), while FKBP52 was detected just above background (not shown). In addition, Hsc/Hsp70 and its co-chaperone DNAJA1 were observed to co-adsorb with Aha1 from HeLa cell extracts (Fig. 2C). However, no detectable HOP (not shown) or Cdc37 was co-adsorbed with Aha1. Furthermore, the putative Hsp90/Aha1 client kinase c-Src did not co-adsorb with Aha1. These results indicate that Aha1 interacts with Hsp90, p23, Hsp70 and DNAJA1 in cells, and that this interaction appears to occur exclusive of the binding of Cdc37 and HOP.

3.3. Interaction of Aha1 with Hsp90–client complexes

3.3.1. Interaction of Aha1 with v-Src

Aha1 enhances v-Src activity in yeast strains deficient in Hsp90 activity, while v-Src activity is suppressed in yeast strains carrying deletions of the *HCH1* and *AHA1* genes [4,5,11], suggesting that the Src kinase is a possible client protein of Hsp90/Aha1. However, no direct interaction of Aha1 with Hsp90/v-Src complexes has been reported. Furthermore, our results, in agreement with work of others [12], suggest that the binding of Cdc37, the "kinase-specific" co-

chaperone, and Aha1 to Hsp90 appears to be mutually exclusive. Therefore, we examined whether Aha1 interacted with molybdate-stabilized complexes formed between Hsp90, Cdc37 and v-Src in RRL. Immunoadsorption of Cdc37 specifically pulled down Hsp90 and v-Src, but not Aha1, while immunoadsorption of His-tagged Aha1 pulled down Hsp90, but did not co-adsorb any v-Src above the levels that non-specifically bound to control resins (Fig. 3A). Thus, there does not appear to be any direct interaction of Aha1 with Hsp90 complexes containing newly synthesized v-Src in RRL: a finding that is consistent with the observation that no c-Scr was detected to be bound to Aha1 immunoadsorbed from extracts of HeLa cells over-expressing Flag-tagged Aha1 (Fig. 2C).

The effect of Aha1 overexpression on v-Src activity was also examined. In the absence of geldanamycin, v-Src matures in RRL yielding an autophosphorylated v-Src band with a slightly slower electrophoretic mobility (Fig. 3B, Upper Panel, lane 2). Maturation of v-Src in RRL containing over-expressed Aha1 did not increase the amount of autophosphorylated v-Src (Fig. 3B, Upper Panel, lane 3). Geldanamycin blocked the maturation of v-Src into an active kinase, whether or not Aha1 was over-expressed (Fig. 3B, Upper Panel, lanes
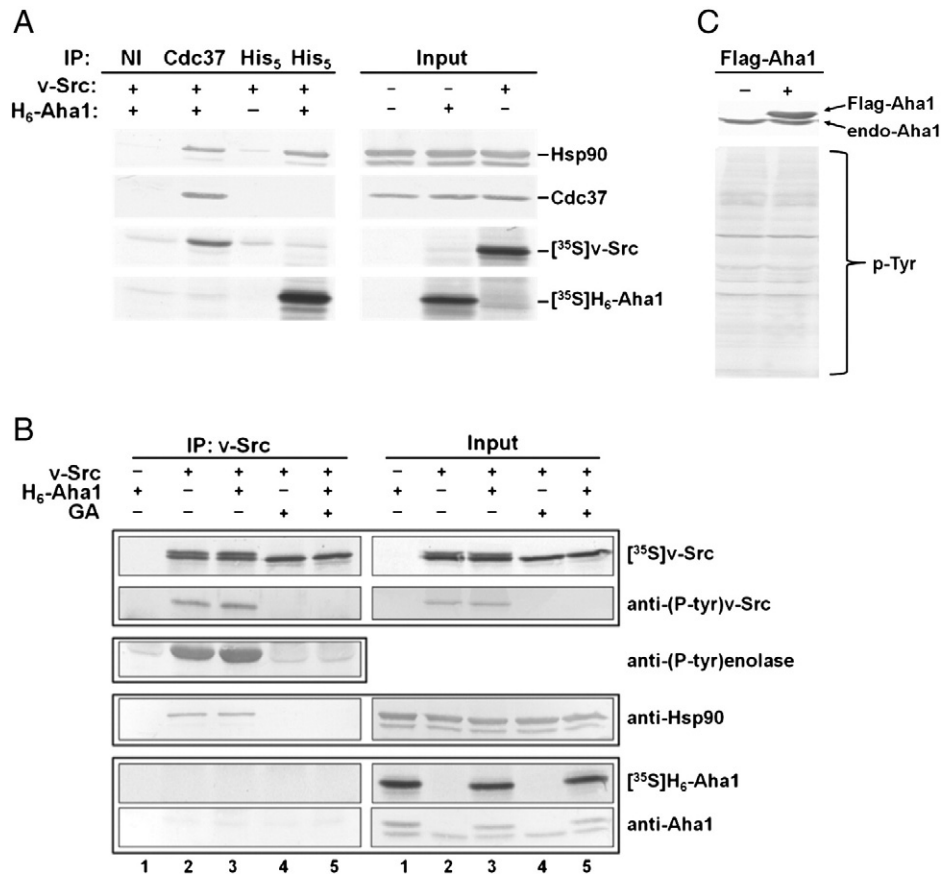

Fig. 3. Aha1 does not associate with Hsp90 chaperone complexes containing bound v-Src or stimulate v-Src activity (A,B), and phosphotyrosine levels are not increased in Aha1 over-expressing HeLa cells (C). (A) [35S]His-tagged Aha1 and [35S]v-Src were synthesized separately by TnT in RRL. The reactions were then combined and incubated for another 20 min with 20 mM molybdate added for the last 5 min. The samples were subjected to immunoprecipitation with anti-Cdc37 or anti-His-tag resins. The immunoresins were washed once with P100T followed by three washes with P50T and analyzed for co-adsorbing protein by SDS-PAGE and autoradiography. Non-immune (NI) mouse IgG resin was control for non-specific binding of Aha1 and TnT reaction containing empty vector (−) was the control for non-specific binding of v-Src. (B) [35S]-Labeled v-Src was synthesized by TnT in RRL with (+) or without (−) 15 µg/ml geldanamycin (GA) at 30 °C for 30 min. Lysate containing radiolabeled v-Src (+) was mixed with RRL containing [35S]Aha1 (+) synthesized as described, followed by an additional 30 min incubation at 30 °C. Control samples were mixed with RRL lacking vector encoding v-Src (−) or Aha1 (−). v-Src was adsorbed with anti-Src antibody. Samples were then washed and the tyrosine kinase activity of immunoadsorbed v-Src was measured by its ability to phosphorylate acid-denatured enolase as described under Section 2.2. Samples were then analyzed by SDS-PAGE followed by autoradiography to examine the interaction between v-Src (Upper Panel) and Aha1(Lower Panel), or Western blotting with anti-phosphotyrosine antibody to measure v-Src autophosphorylation (Upper Panel) and tyrosine kinase activity, and anti-Hsp90 and anti-Aha1 (Lower Panel) to examine the co-adsorption of these proteins with v-Src. Input: samples prior to immunoadsorption of v-Src. (C) Over-expression of Aha1 did not alter the total phosphotyrosine level in HeLa cells. Cultured HeLa cells were transfected with a pcDNA3.1 vector expressing Flag-Aha1 or empty pcDNA3.1 vector using Lipofectamine 2000 transfection reagent. After 48-hour incubation at 37 °C, cells were lysed by RIPA lysis buffer containing protease and phosphatase inhibitor cocktails, followed by the addition of SDS-sample buffer. Samples were then boiled for 5 min, followed by analysis of the samples by 8% SDS-PAGE and Western blotting. The endogenous and exogenous Aha1 levels were detected by blotting with rabbit polyclonal anti-Aha1 antibody and the phosphotyrosine levels in whole cell lysates were detected by the Western blot with an anti-phosphotyrosine monoclonal antibody mixture (Cell Signaling, P-Tyo-100, Cat #9411; and P-Tyo-102, Cat #9416; 1:1 mixture).

4 and 5). Western blotting with anti-phosphotyrosine antibody, confirmed that v-Src's electrophoretic mobility shift was due to its autophosphorylation, and indicated that over-expression of Aha1 did not stimulate v-Src's autophosphorylation activity (Fig. 3B, anti-(P-tyr)v-Src). In addition, overexpression of Aha1 did not stimulate v-Src's tyrosine kinase activity as measured by the ability of immunoadsorbed v-Src to phosphorylate acid-denatured enolase in vitro (Fig. 3B, anti-(P-try)enolse). As expected, geldanamycin blocked the interaction of Hsp90 with v-Src (Fig. 3B, anti-Hsp90) and v-Src autophosphorylation (Fig. 3B, Upper two panels). The no plasmid control, and maturation of v-Src in the presence of geldanamycin confirmed that the phosphotyrosine bands detected by Western blotting were specific to the presence of active v-Src (Fig. 3B). Again, no Aha1 was detected co-immunoadsorbing with v-Src (Fig. 3B, Lower panel). Thus, a direct assay of v-Src tyrosine activity in lysate containing elevated levels of Aha1 (~3 times greater than levels of endogenous Aha1) indicated that Aha1 had no effect on v-Src's activity.

3.3.2. Interaction of Aha1 with the progesterone receptor

Similarly, we tested the hypothesis that the reported ability of Aha1 to stimulate SHR function was a result of its direct interaction with Hsp90/PR complexes. Immunoadsorption of Hsp90 from lysate expressing [^{35}S]PR and His-tagged [^{35}S]Aha1 coadsorbed both PR and Aha1, and geldanamycin was found to decrease the association of both of these proteins with Hsp90 (Fig. 4A). However, analysis of anti-His-tag pull downs of Aha1 indicated that no [^{35}S]PR was present in Hsp90/Aha1 complexes stabilized with molybdate in RRL (Fig. 4B). Thus, similar to the results obtained with v-Src, Aha1 was absent from complexes formed between Hsp90 and PR, a member of another major family of Hsp90-dependent clients.

3.3.3. Interaction of Aha1 with Akt

The co-adsorption of Aha1 with the Hsp90 client Akt has been reported from lysates of cultured HeLa cells [4,5]. To investigate this reported interaction, HeLa cells were transfected with Flag-tagged Aha1, lysed and immunoadsorbed with anti-Flag antibody. Western blotting indicated that Akt was specifically co-adsorbed with Aha1 (Fig. 5A). To determine the effect of Aha1 on Akt activity, Flag-tagged Aha1 or empty vector was transfected into HeLa cells. Akt was immunoadsorbed with anti-Akt/pS473 antibody and its ability to phosphorylate glycogen synthetase kinase 3 (GSK3) was assayed. While Western blot analysis with anti-Akt antibody indicated that equivalent amounts of Akt were present in the cell lysates, Western blotting with anti-Akt/pS473 indicated that Akt from Aha1 over-expressing cells had a decreased level of this activating phosphorylation (Fig. 5B, lysate). Consistent with this observation, the phospho-Akt immunoadsorbed from Aha1 over-expressing cells

had a decreased ability to phosphorylate GSK3 (Fig. 5B: IP). Thus, in contrast to the previous report [21], we found that over-expression of Aha1 decreased rather than increased the activity of Akt in HeLa cells.

Since, neither v-Src expressed in RRL, nor HeLa cell c-Src could be detected to interact with Aha1, we also examined whether Aha1 was present in Hsp90 complexes formed with nascent Akt synthesized in RRL [39]. Both Hsp90 and Cdc37 were observed to co-adsorb with nascent His-tagged Akt (Fig. 5C). While binding of Hsp90 to newly synthesized Akt was maintained in the presence of geldanamycin, albeit at a reduced level, geldanamycin completely blocked the binding of Cdc37 to Akt. When the Akt was matured in RRL for a further 1.5 h, its interaction with Hsp90 and Cdc37 was decreased (Fig. 5C versus 5D). However, under conditions where addition of 20 mM sodium molybdate stabilized the binding of Hsp90 and Cdc37 with Akt (Fig. 5D), Western blotting with anti-Aha1 antibody did not detect any Aha1 associated with Akt. Thus, similar to results obtained with nascent v-Src, Aha1 does not appear to bind to complexes formed between Hsp90 and nascent Akt in RRL.

3.4. Effect of Aha1 overexpression on client protein activity

Because overexpression of Aha1 stimulated the activity of v-Src and SHR in yeast strains deficient in Hsp90 function [4,5,12], it has been generally assumed that stimulation of Hsp90's ATPase activity would increase its ability to chaperone client folding, and thus, enhance client protein activity. To test this hypothesis, we examined the effect that over-expression of Aha1 had on total cellular tyrosine kinase activity by determining overall phosphotyrosine levels in HeLa cells by Western blotting. Expression of Aha1 at a level approximately 5-times higher than that of its endogenous expression, caused no change in the level of tyrosine phosphorylation in HeLa cells (Fig. 3C). Thus, marked overexpression of Aha1 had no effect on the overall tyrosine kinase activity in cultured HeLa cells.

To further test this hypothesis, we examined the effect of over-expression of Aha1 on Hsp90-dependent refolding of thermally denatured luciferase in RRL [24]. TnT-RRL was incubated in the presence and absence of plasmid encoding Aha1, and the ability of the lysates to refold denatured luciferase was measured after 30 min. Western blotting with anti-Aha1 antibody indicated that there was approximately a 3-fold higher level of Aha1 in the RRL programmed with Aha1 plasmid than in the control (not shown). Contrary to expectations this increase in the expression of Aha1 resulted in a statistically significant ~40% decrease in the rate of luciferase refolding compared to the control (Fig. 6). Similar results were obtained upon titration of purified recombinant Aha1 into RRL (not shown). Thus, increasing Aha1 levels does not seem to generally enhance Hsp90's "chaperone activity".
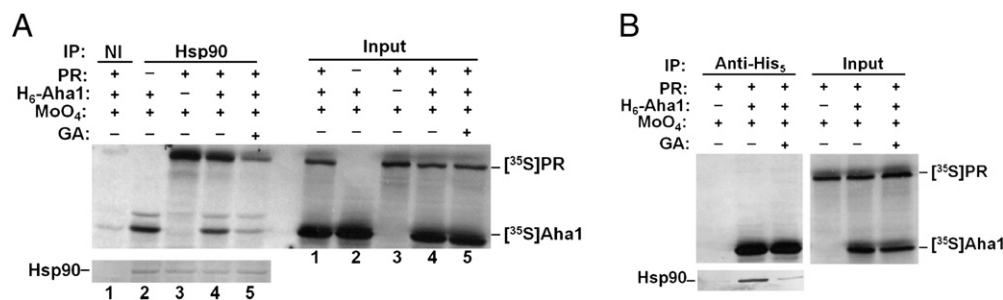

Fig. 4. Aha1 does not interact with Hsp90 complexes containing the progesterone receptor. [^{35}S]-Labeled PR was synthesized by TnT in RRL with or without 15 μg/ml geldanamycin (GA) at 30 °C for 30 min. To examine steroid hormone receptor binding activity of Aha1, lysates containing radiolabeled PR (+) were mixed with RRL containing [^{35}S]Aha1 (+) synthesized as described above, followed by an additional 30 min incubation at 30 °C. Control samples were mixed with RRL lacking vector encoding PR (−) or Aha1 (−). Hsp90 (A) or His-tagged Aha1 (B) was adsorbed with anti-Hsp90 or anti-His-tagged antibody in the presence of 20 mM molybdate and samples were washed and analyzed by SDS-PAGE followed by Western blotting and autoradiography as described under Section 2.1. NI—Nonimmune IgG control for non-specific binding.
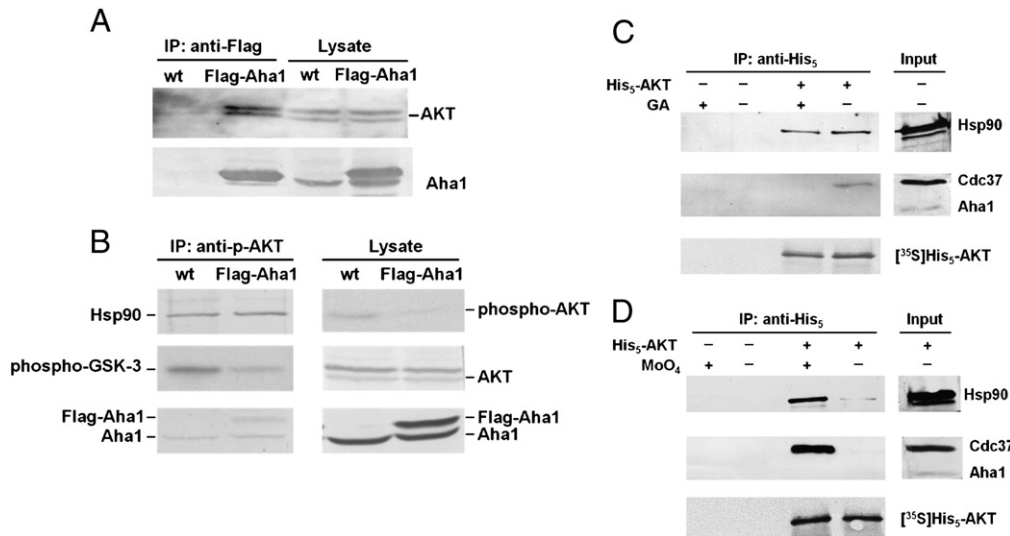
Fig. 5. Interaction of Aha1 with AKT in HeLa (A,B) cells and RRL (C,D). (A) Empty control vector (wt) or vector for the expression of Flag-tagged Aha1 was transfected into HeLa cells and cell extracts were immunoadsorbed with anti-Flag agarose. Co-immunoprecipitated AKT was detected by Western blotting. (B) Active AKT was immunoprecipitated from lysates of control or Aha1 over-expressing HeLa cells and assayed in vitro for kinase activity using GSK-3 fusion protein as the substrate of AKT as described under Section 2.3. Coadsorbing Hsp90 and Aha1 and the level of phospho-AKT, total AKT and Aha1 in the HeLa cell extracts were detected by Western blotting. AKT catalyzed phosphorylation of GSK3 was detected by Western blotting. (C,D) Aha1 does not interact with newly synthesized AKT in RRL. (C) To assess the interaction of endogenous Aha1 with Akt, [^{35}S]-His-tagged Akt was generated by TnT in RRL for 20 min at 37 °C, followed by an additional 10 min incubation after the addition of 10 μM GA.. [^{35}S]-Aha1 was immunoadsorbed with anti-His antibody, and the resin was washed once with P60T, twice with P100T and once with P60T, followed by analysis of the samples by SDS-PAGE, Western blotting and/or autoradiography. (D) To assess the interaction of endogenous Aha1 with matured Akt, [^{35}S]-His-tagged Akt was generated by TnT for 30 min at 30 °C, followed by the addition of 20 mM sodium molybdate (+) or buffer control (−). After an additional incubation for 1.5 h, [^{35}S]-Aha1 was immunoadsorbed with anti-His antibody. Since the interaction of Hsp90 and its associated components is affected by the salt concentration in the wash buffers, the NaCl concentrations in the wash buffer were reduced by 60 mM to control for the ionic strength of 20 mM Na$_2$MO$_4$ contained in the wash buffers for the molybdate treated samples. Samples were washed as described above and analyzed by SDS-PAGE, Western blotting and/or autoradiography. (−) No AKT plasmid.

3.5. Identification of Aha1-interacting proteins

Since Aha1 could not be detected to interact with v-Src or the PR, which studies in yeast suggested were putative clients, we ask the question, to what proteins in HeLa cells does Aha1 interact? Flag-Aha1 or empty vector was transfected into HeLa cells, and cell extracts were prepared after 48 h. Extracts were adsorbed with anti-Flag agarose, and bound proteins were eluted and separated by SDS-PAGE. Samples were either analyzed directly by Western blotting or gel bands were excised, digested with trypsin and analyzed by LC–MS/MS (Supplementary Fig. S1). While Western blotting confirmed that SRPK1 [20] was coadsorbed with Aha1 (Fig. 7), an interaction between GTP cyclohydrolase I and Aha1 [22] was not detected (not
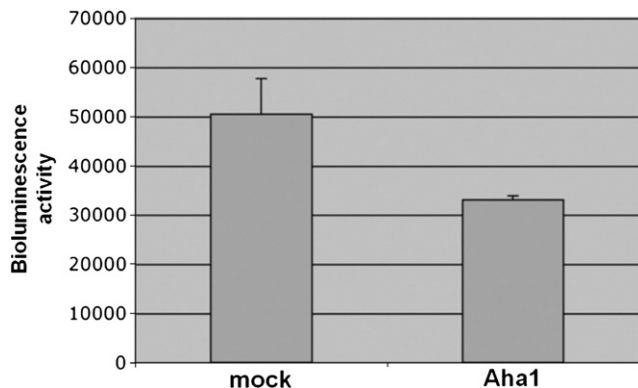
shown). LC–MS/MS identified DNA-activated protein kinase (catalytic subunit), Topoisomerase IIα, Ku80, Ku70, IQGAP1, RACK1, PKN2 protein kinase (a.k.a., PRK2), and PPM1G (a.k.a., PP2Cγ and protein phosphatase 1C) as proteins that co-adsorbed with Aha1. The proteins were absent from the gel lane containing proteins from anti-Flag immuno-adsorption of extracts from HeLa cells transfected with empty vector. The specificity of the interactions of these proteins with Aha1 was further confirmed by Western blotting (Fig. 7).
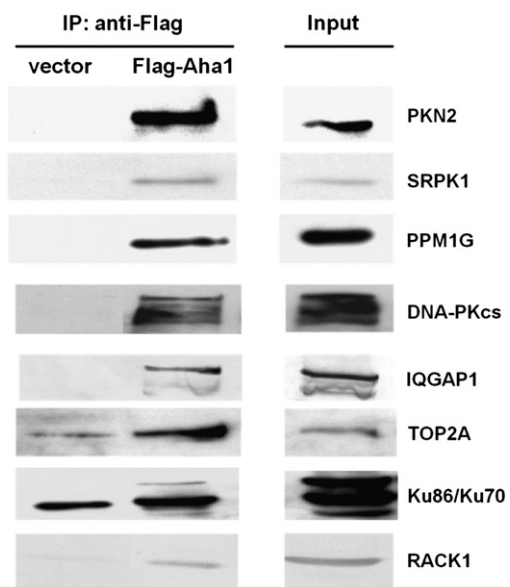

Fig. 6. Over-expression of Aha1 inhibits Hsp90-dependent luciferase renaturation. RRL containing newly-synthesized Aha1 or mock plasmid control was incubated with thermally denatured firefly luciferase at 30 °C for 10 min. The difference in luciferase activity (light units) assayed before and after a 10 min renaturation is plotted. The values represent the average of 3 independent experiments with a P-value (*t* test) of 0.049.

Fig. 7. Confirmation of Aha1 interacting proteins identified by LC–MS/MS. HeLa cell lysates from cells transfected with empty vector or vector encoding Flag-Aha1 were immunoadsorbed with anti-Flag resin and analyzed by SDS-PAGE and Western blotting for the presence of the co-adsorbed proteins indicated in the figure.

4. Discussion

4.1. Co-chaperone components of Hsp90/Aha1 complexes

Aha1 was found to interact with Hsp90 co-chaperone complexes containing bound Hsp70 and DNAJ1, components of "intermediate" Hsp90 client complexes, and p23 and FKBP52, which are components of "late" Hsp90 client complexes (reviewed in [1–3,34]). In addition, no interaction of Aha1 with Hsp90 complexes containing Cdc37 or HOP/Sti1 was detected. Recently, it has been reported that Hsp90 forms "mixed" complexes containing components of both intermediate and late complexes, during Hsp90's transition between these two states [40]. The ability of molybdate to stabilize Aha1's interaction with Hsp90 suggests that Aha1 prefers to interact with the conformation that Hsp90 assumes upon formation of late complexes. Thus, the data suggest that either a mixture of early (Hsp90/Hsp70/DNAJ1) and late (Hsp90/FKBP52/p23) Hsp90 complexes containing Aha1 was pulled down, or that Aha1 is capable of binding to mixed Hsp90 complexes that are formed during its transition between these two states.

With respect to p23/Sba1 binding, structural studies suggest that the binding sites for p23 and Aha1 on Hsp90 overlap [7,41]. However, p23 has been reported to bind at a stoichiometry of one molecule of p23 per Hsp90 dimer [8,42]. In addition, the detection of an Hsp90–Aha1–p23 ternary complex is consistent with a recent report that the binding of one Aha1 molecule per Hsp90 dimer is sufficient to fully activate Hsp90's ATPase activity by spanning the two subunits [10]. Thus, p23 and Aha1 could coexist in the same chaperone complex by binding to opposite faces on the Hsp90 dimer. Still it should be noted that there is some controversy on whether p23/Sba1 binds to Hsp90 at a ratio of one or two molecule(s) per Hsp90 dimer [8,41,43,44], with one report noting that the stoichiometry of p23/Sba1 bound per Hsp90 dimer obtained was dependent upon the ratio of p23 to Hsp90 utilized in the experiment [45].

These results are both in agreement and at odds with previous reports published in regard to the co-chaperones with which Aha1 can coexist in Hsp90/co-chaperone complexes. While there is agreement that Aha1 can bind to Hsp90 complexes containing immunophilins (e.g., the cyclophilin 40 homologs Crp6 and Crp7, [5,12]), there has been disagreement on whether Aha1 can interact with Hsp90 complexes containing bound HOP/Sti1, Cdc37 or p23/Sba1 [5,8,12,43]. As discussed by Gaiser et al. [43], the discrepancy between the results obtained may be due to several causes: 1) the use of a mixed human Cdc37/yeast Hsp90 system [5,8,12]; 2) the utilization of truncated HOP/Sti1(cSti1), and Cdc37 (Cp50) Hsp90 binding constructs [8]; or 3) the employment of different experimental techniques [5,8,12,43]. It is also noteworthy that the binding properties of yeast and human Cdc37 to Hsp90, as well as the regulation of Cdc37–Hsp90 complexes by nucleotides and other co-chaperones differ markedly between the yeast and human systems [43]. Thus, Hsp90/co-chaperone interactions that are mutually exclusive in human cells may not be mutually exclusive in yeast. It should also be noted that these previous reports were based on work done with purified recombinant proteins in vitro, which may yield results different from those obtained here using cell extracts.

4.2. Effect of Aha1 on Hsp90–client protein activity

The observation that over-expression of Aha1 in HeLa decreased the level of activation of Akt, while increased expression of Aha1 in RRL inhibited rather than stimulated Hsp90-dependent refolding of denatured luciferase, indicates that stimulating Hsp90's ATPase activity may not in general stimulate Hsp90's ability to facilitate protein folding or activation. This observation is consistent with a model recently proposed for the role of Aha1 in the Hsp90-dependent maturation of wild type and mutant/ΔF505 CFTR [9]. In this model,

the interaction of Aha1 with Hsp90/CFTR complexes is postulated to decrease the dwell time of Hsp90 in CFTR complexes, stimulating the dissociation of Hsp90 from immature, misfolded wild-type or mutant/ΔF505 CFTR, and the formation of CFTR/Hsp70/DNAJ complexes, thus inhibiting the translocation of the proteins to the plasma membrane [9,15,46]. Ultimately, Aha1 stimulated dissociation of Hsp90 led to the ubiquitination of immature or mutant CFTR and its degradation though the ERAD pathway or targeting to the lysosome [9,15,46]. It was proposed that decreased dwell time of Hsp90 in complexes with clients due to Aha1 stimulated ATP hydrolysis can interfere with maturation or folding of certain clients, but may stimulate the maturation of other clients (e.g., v-Src) [9]. Such a role for Aha1 in modulating Hsp90/client interactions is also consistent with the observation that Aha1 negatively regulates the activity of the MAL activator in *Saccharomyces* by stimulating the dissociation of Hsp90 from the MAL activator prior to its complete maturation [23]. In addition, over-expression of Aha1 has also been observed to increase total MC4R levels in cells, but decrease the amount of MC4R that is translocated to the plasma membrane [18]. Such a mechanism of action would facilitate the turnover of mutant and/or misfolded proteins that might otherwise have extended interactions with Hsp90 and interfere with cellular proteostasis [9].

Our results suggest that the stimulatory effect that Aha1 has been reported to have on the activity of v-Src and SHRs in yeast is likely to be an effect down-stream of Hsp90-dependent folding and maturation of the newly synthesized proteins. While genetic evidence indicates that over-expression of Aha1 in Hsp90 deficient yeast restores the activity of exogenously expressed v-Src and enhances the activity of ectopically expressed SHR [12], no interaction of Aha1 with Hsp90 complexes formed with newly synthesized v-Src or PR was observed in RRL, and no interaction of Aha1 with endogenous c-Src could be detected in HeLa cells. Furthermore, over-expression of Aha1 in RRL had no stimulatory effect on the kinase activity of v-Src, and Aha1 overexpression in HeLa cells had no effect on cellular phosphotyrosine levels. These results suggest that the effects of Aha1 over-expression on the activity of v-Src and SHRs in yeast are either indirect or mediated through Aha1's interaction with Hsp90 complexes formed with client proteins after their maturation. These possibilities would be consistent with the observation that the interaction of Cdc37 and HOP/Sti1 (co-chaperones that are required for the maturation of kinases and SHRs) with Hsp90 appears to be mutually exclusive of Aha1. Still the absence of a detectable interaction between Aha1 and Hsp90/v-Src or Hsp90/PR complexes might be due to its role in stimulating the dissociation of Hsp90/client complexes, and that Aha1's interaction with such complexes may be so transient that we fail to detect it. However, we note that no interaction of Aha1 was observed with Hsp90/v-Src or PR complexes that were stabilized by the addition of molybdate, a condition which also stabilizes the binding of Aha1 to Hsp90. In addition, another caveat needs to be acknowledged. Because of distinct differences in: 1) the ATPase activities of yeast and human Hsp90; 2) the interactions of yeast and human Hsp90 with their co-chaperones; and 3) the effect of these co-chaperone interactions on Hsp90's ATPase activity (discussed in [43]), Aha1 may have a direct Hsp90-dependent effect on v-Src activity in yeast, but this effect on v-Src activity may not be extrapolatable to human cells.

4.3. Identification of Aha1 interacting proteins

Because the results discussed above suggested that Aha1 may not function as a "generic" co-chaperone associated with the folding and maturation of Hsp90-dependent nascent polypeptides, studies were initiated to begin to identify proteins that specifically interacted with Aha1 in HeLa cells. SRPK1, DNA-PKcs, Topo IIα, Ku80/Ku70, IQGAP1, RACK1, PKN2 protein kinase, and PPM1G were identified as proteins that interact with Aha1. DNA-PKcs, Topo IIα and Ku80/Ku70 function

in numerous cellular processes, including double-stranded DNA repair and telomere maintenance [47,48]. DNA-PKcs [49] and Topo IIα [50,51] have previously been demonstrated to be present in multi-meric complexes with Hsp90, while Ku80/Ku70 is a multi-functional protein complex that acts as the regulatory subunit of DNA-PKcs [47,48,52]. SRPK1 [20] and PPM1G [53] phosphorylate and dephosphorylate factors involved in the assembly and regulation of the spliceosome, respectively. Stress-induced translocation of SRPK1 into the nucleus is accompanied by the dissociation of Hsp90, and functions to regulate alternate splicing of transcripts that occurs in response to stress [20]. IQGAP1 is a scaffold protein that integrates diverse signaling pathways, including pathways involved in regulating cell proliferation, cell adhesion, and cytoskeletal rearrangements and cell motility [54,55]. IQGAP1 interacting proteins include the Hsp90-dependent protein kinases EGFR, c-Src, B-Raf, Akt, and MEK1 [54–56]. RACK1 (receptor of activated protein kinase C) is another multi-functional scaffold protein [57], one function of which is to regulate the O_2-independent degradation of HIF1α by competing with Hsp90 for the binding of HIF1α [58,59]. In addition, RACK1 acts as a scaffolding protein that functions to modulate CFTR activity [60–63]. PKN2 is a protein kinase C-related protein kinase that plays an essential role in regulating entry into mitosis and exit from cytokinesis [64]. PKN2 has been shown to regulate nuclear translocation of a subset of class IIa histone deacetylases [65], and to interact with PDK1, a Hsp90-dependent protein kinase that modulates signaling through Akt [66]. Thus, a common feature of the proteins that were found to interact with Aha1 is that they are part of or regulate the assembly of oligomeric complexes that contain components that interact with Hsp90.

5. Conclusion

In summary, our data suggest that Aha1 may not be a generic co-chaperone of Hsp90 that generally functions to modulate the Hsp90-dependent folding of nascent polypeptide chains. Rather Aha1 may play a role in protein sorting or quality control at membranes, as suggested from its role in modulating the turnover of CFTR [9,14,15], the transport of VSV-G [13], and its interaction with activated Akt ([21] and Fig. 5). In addition, consistent with the observation that Aha1 expression is stress-inducible [5], our data suggest that Aha1 may function in cellular stress responses, such as regulating proteins involved in DNA repair, and alternate splicing of mRNAs [20] that are induced by DNA damage and other environmental stresses [67].

Abbreviations

Hsp	heat shock protein
90 kDa Hsp	Hsp90
Hsp70	used to generically refer to all members of the Hsp family
Aha1	activator of Hsp90 ATPase
Cdc37	generically used to refer to the protein product of *CDC37* gene homologs regardless of source of organism
p23/Sba1	the mammalian or yeast equivalent of the co-chaperone p23
HOP/Sti1	the mammalian or yeast equivalent of the Hsp90–Hsp70 organizing protein
HIP	Hsp70-interacting protein
Cyp40	cyclophilin 40
Cpr	yeast cyclophilin 40-homolog
PP5	protein phosphatase-5
DNAJ	eukaryotic DnaJ homolog
TnT	coupled transcription/translation of protein
RRL	rabbit reticulocyte lysate
IgG	immunoglobulin G
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
PIPES	piperazine-N,N′-bis[2-ethanesulfonic acid]
MS/MS	tandem MS
FKBP	FK506 binding protein
DMSO	dimethylsulfoxide
PVDF	polyvinylidene difluoride
VSV-G	vesicular stomatitis virus glycoprotein
CFTR	cystic fibrosis transmembrane conductance regulator
GR	glucocorticoid receptor
PR	progesterone hormone receptor
SHR	steroid hormone receptor
GA	geldanamycin
FT	Fourier transformed
MS	Mass Spectroscopy
LC	liquid chromatography
ECL	enhanced chemiluminescence
TCEP	tris-2-carboxyethyl-phosphine
ER	endoplasmic reticulum
ERAD	ER-associated protein degradation

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbamcr.2012.03.014.

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