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Activation of the ATPase Activity of Hsp90 by the Stress-Regulated Cochaperone Aha1

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Summary

Client protein activation by Hsp90 involves a plethora of cochaperones whose roles are poorly defined. A ubiquitous family of stress-regulated proteins have been identified (Aha1, activator of Hsp90 ATPase) that bind directly to Hsp90 and are required for the in vivo Hsp90-dependent activation of clients such as v-Src, implicating them as cochaperones of the Hsp90 system. In vitro, Aha1 and its shorter homolog, Hch1, stimulate the inherent ATPase activity of yeast and human Hsp90. The identification of these Hsp90 cochaperone activators adds to the complex roles of cochaperones in regulating the ATPase-coupled conformational changes of the Hsp90 chaperone cycle.

Introduction

Hsp90 is an essential cellular chaperone, involved in the activation of key signaling and regulatory systems in eukaryotic cells (Pearl and Prodromou, 2002). Rather than acting at an early stage of folding, Hsp90 binds client proteins in a substantially folded form and facilitates their association with cofactors or other proteins required for full activity. Hsp90-dependent activation of CPs in vivo involves a plethora of cochaperones, which associate with the Hsp90-based complex at different stages of the activation process (reviewed in Pratt, 1998). Thus, in the activation of steroid hormone receptors (SHRs) p60/Hop is present in Hsp90-receptor complexes at an early stage prior to activation. In contrast, p23 and the TPR-domain immunophilins, such as Cyp40, are associated with the mature complexes in which the receptor is capable of binding steroid hormone. At least in some cases, cochaperone association is also client specific, such that Hsp90 complexes with protein kinases contain p50/Cdc37, whereas those with SHRs do not.

At least six Hsp90 cochaperones have been identified (Hop/Sti1, p50/Cdc37, Cns1, p23/Sba1, Cpr6, and Cpr7); however, the role(s) played by most of them remains obscure. Hop and its yeast homolog Sti1 act as scaffolds, binding to Hsp70 via its N-terminal TPR domain and to Hsp90 via its central and/or C-terminal TPR domain(s) (Chen et al., 1996). Additionally, Hop/Sti1 was found to downregulate the ATPase activity of Hsp90, providing further evidence for regulation of the ATPase activity at different stages of the CP activation process (Prodromou et al., 1999).

Here we describe the identification of Aha1, the prototype of a ubiquitous family of stress-regulated Hsp90 cochaperones, which are required for stress-tolerance and participate in Hsp90-dependent CP activation in yeast. In vitro, yeast Aha1 and its shorter homolog Hch1 are Hsp90 binding proteins that activate the inherent ATPase activity of Hsp90. Modulation of Hsp90s ATPase activity by Aha1 and other cochaperones may regulate progress through the chaperone cycle and thereby control the efficiency of Hsp90-dependent CP activation.

Results

Identification of the Hch1/Aha1-Protein Family

Homology searches with *HCH1* (153 amino acids), a high-copy-number suppresser of a Hsp90 *ts*-mutation (E381K) (Nathan et al., 1999), revealed the yeast open reading frame (ORF), YDR214W (designated *AHA1*). *AHA1* encoded a hypothetical 350 residue protein whose N-terminal half had 36.5% sequence identity to Hch1 but with an additional 197 residues at the C terminus. Sequences with similarity to the Aha1 sequence over its entire length could be identified in organisms from yeast to humans. Only one other example of the shorter Hch1-like sequence was identified in the yeast *C. albicans* (Figure 1A). Two genes encoding clear homologs of Aha1 were identifiable in the draft human genome, one mapping to chromosome 2 (Ensembl gene 00000152645) and the other to chromosome 14 (Ensembl gene 00000100591, now designated hAha1). Analysis of EST databases suggests the latter is highly

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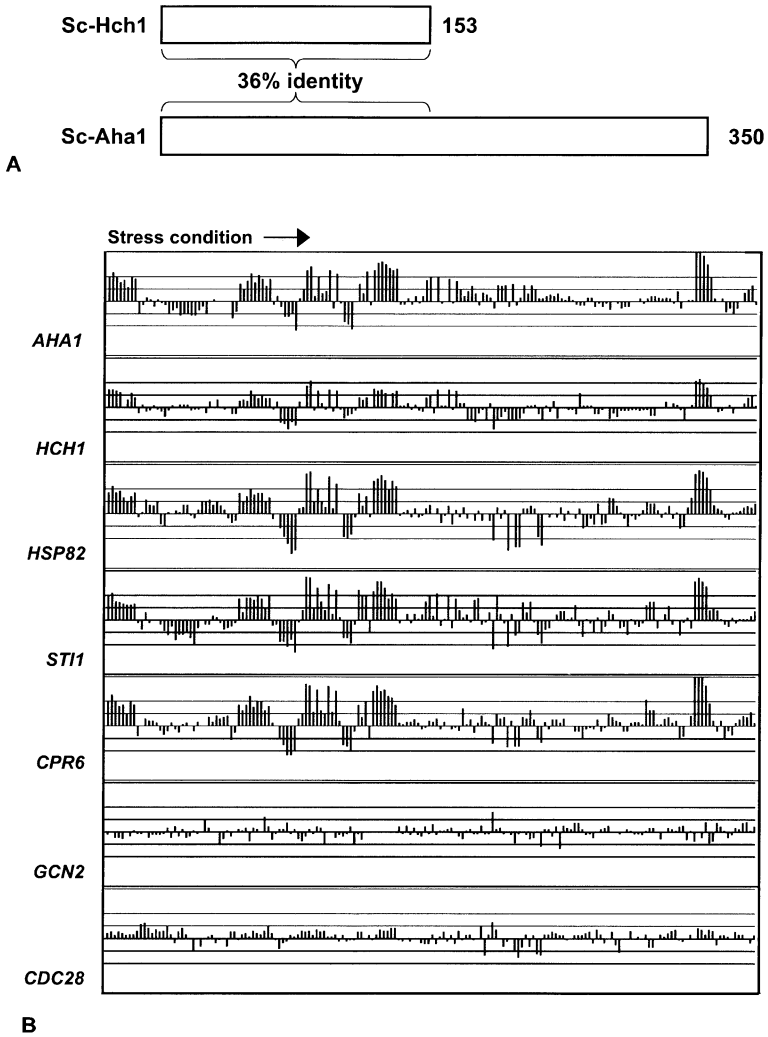

Figure 1. Domain Structure and Expression Profiles of Aha1 and Hch1

(A) Homologs of the 350 residue *S. cerevisiae* Aha1 protein (Sc-Aha1) were identified in *A. thaliana*, *D. melanogaster*, *C. elegans*, *H. sapiens* (chromosome 14, Ensembl gene 00000100591), and *S. pombe,* while a homolog of the shorter 153 residue Hch1 protein was only identified in *C. albicans*. Sequence identity among Aha1 homologs varies between 19.7%–38.3%. The Hch1 protein and N-terminal domain of Aha1 have 36.5% sequence identity.

(B) Expression profiles for *S. cerevisiae* Hsp90 (*HSP82*) and the known cochaperones *CPR6* and *STI1* match closely those of *AHA1* and *HCH1* while those of Hsp90 client proteins (e.g., *GCN2* and *CDC28*) are very different. Each vertical bar represents a different stress condition, with bars above the center line representing upregulation, those below representing downregulation, and the length of the bar reflecting the degree of change in expression. This figure was adapted from Gasch et al. (2000), and the exact conditions of stress can be found in that publication.

expressed in most tissues. Database searches using PSI-BLAST failed to identify any distant homologs of known function, and sequence threading failed to identify any known protein fold within Aha1/Hch1 sequences, from which their function might be inferred.

Stress Regulation of HCH1, and AHA1 and Its Human Homolog

Its original isolation as a suppresser of an Hsp90 *ts*-mutation (Nathan et al., 1999) raised the possibility that *HCH1*, and consequently *AHA1*, might encode previously uncharacterized cochaperones of the Hsp90 complex. This notion is supported by the behavior of *HCH1 AHA1* genes in response to various stress conditions (Gasch et al., 2000). Both genes display patterns very similar to *HSP82* and cochaperones such as *STI1* and *CPR6* but quite different from the Hsp90 clients, *GCN2* and *CDC28* (Figure 1B). Consistent with their patterns of stress regulation, *AHA1* and *HCH*1 promoters contain consensus heat shock-factor elements (*HCH1*: −165 to −157, TTCTAGAA; *AHA1*: −363 to −356, GAA TATTC; −301 to −294, GAATTTTC; and −105 to −98, GAAAGTT). We have previously shown that treatment of mammalian cells with the geldanamycin (GA) derivative 17-allylamino, 17-demethoxygeldanamycin (17AAG), an Hsp90 inhibitor, increased expression of several heat shock and stress-regulated genes, including Hsp70s and Hsp90 (Clarke et al., 2000). In contrast, levels of CPs, such as Raf-1 and Akt/PKB, decrease (Hostein et al., 2001). Expression array analysis of *hAha1* shows clear upregulation in response to 17AAG treatment or heat shock, as observed for other chaperones/cochaperones (Figures 2A and 2B). In support of this, anti-yeast Aha1 polyclonal serum crossreacted with a band of the correct relative molecular weight whose intensity increases following 17AAG or heat shock treatment of HT29 cells (Figure 2C). Subsequently, an upregulated protein identified in 2D gels of 17AAG-treated A2780 cells (Figure 2D) was identified as hAha1 by MALDI-MS peptide mass mapping. Increased levels of the Aha1-homolog was fully consistent with the behavior of known chaperones/cochaperones (Clarke et al., 2000) and suggest that, like its yeast counterpart, at least one human Aha1 homolog is also subject to stress regulation.

Dependence of Cell Growth and Client Protein Activation on Aha1 and Hch1

If Aha1 and Hch1 are cochaperones of the Hsp90 system, then deficiencies in these might affect the function of the Hsp90 system in yeast. To test this, we con-

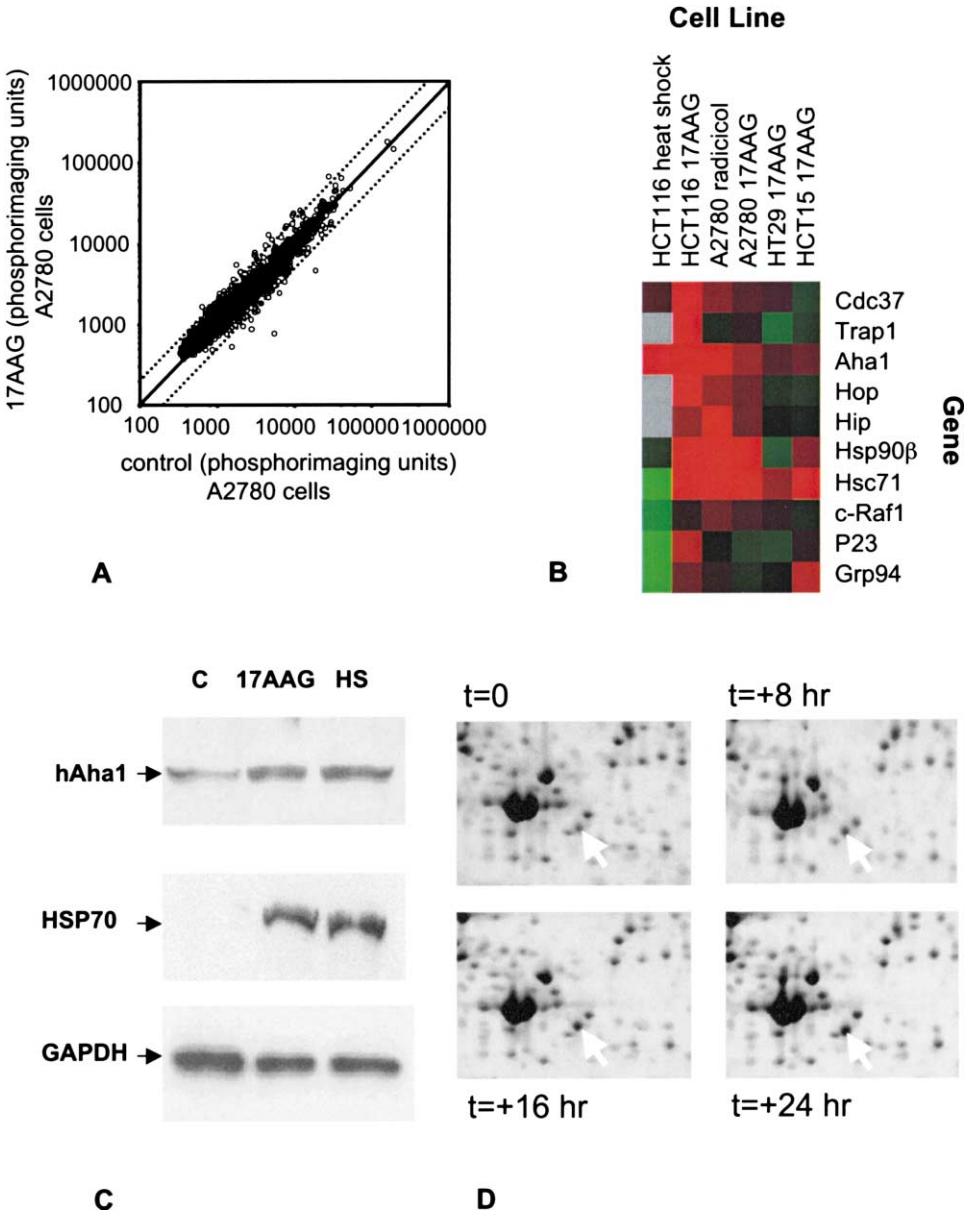

Figure 2. Microarray and Proteomic Analysis of Human Aha1 Expression

(A) Scatter plot of control gene expression versus 17AAG treated (60 nM) in A2780 cells. Each point is a single gene; a 1:1 ratio is expected if expression is unchanged by treatment (central line). Outer dotted lines mark a 2-fold change in expression. The full microarray profile is available as supplemental data at http://www.molecule.org/cgi/content/full/10/6/1307/DC1.

(B) Changes of gene expression in human colorectal (HCT116, HCT15, and HT29) and ovarian (A2780) cancer cells treated for 24 hr with Hsp90 inhibitors (60 nM 17AAG or 0.6 μM radicicol) or heat shock (HS). Red indicates increase by treatment; green, a decrease; black, no change; and gray, a missing value. hAha1 expression increases strongly following heat shock and to a lesser extent with 17AAG or radicicol treatment.

(C) Western blot of HT29 cells treated with either 17AAG (50 nM) or heat shock (HS). C, untreated cells. A band corresponding in size to the expected hAha1 and crossreacting with anti-yAha1 Abs is detected (indicated by the arrow) and is seen to increase with 17AAG and HS treatment. Similarly, Hsp70 expression increases with 17AAG and HS treatment as previously observed (Clarke et al., 2000). GADPH is the loading control.

(D) Two-dimensional gels showing induction of hAha1 protein (indicated by the arrow) in A2780 control cells and after 8, 16, and 24 hr of 17AAG treatment. Results show that hAha1 is induced following treatment. Paired gels were run for each time point, with four gels at time 0. The mean relative intensities at each time point with respect to time 0 were 1.2 at 8 hr, 1.8 at 16 hr, and 2.0 at 24 hr. The M_r and PI of Aha1 estimated from the gel were 41 and 5.44 kDa, respectively. MALDI mass spectrometry following tryptic in-gel digests of the upregulated spot identified fourteen species matching tryptic peptides of hAha1. Sequence coverage was 55%, and all peptides matched the DNA-derived hAha1 sequence.

structed strains deleted for *AHA1* (*Δaha1*), *HCH1* (*Δhch1*), or both (*Δaha1-Δhch1*) and analyzed their growth characteristics. Under normal conditions all three strains were viable, indicating that Aha1 and Hch1 are not essential components of the Hsp90 system. However, *Δaha1* and the double-delete strain displayed

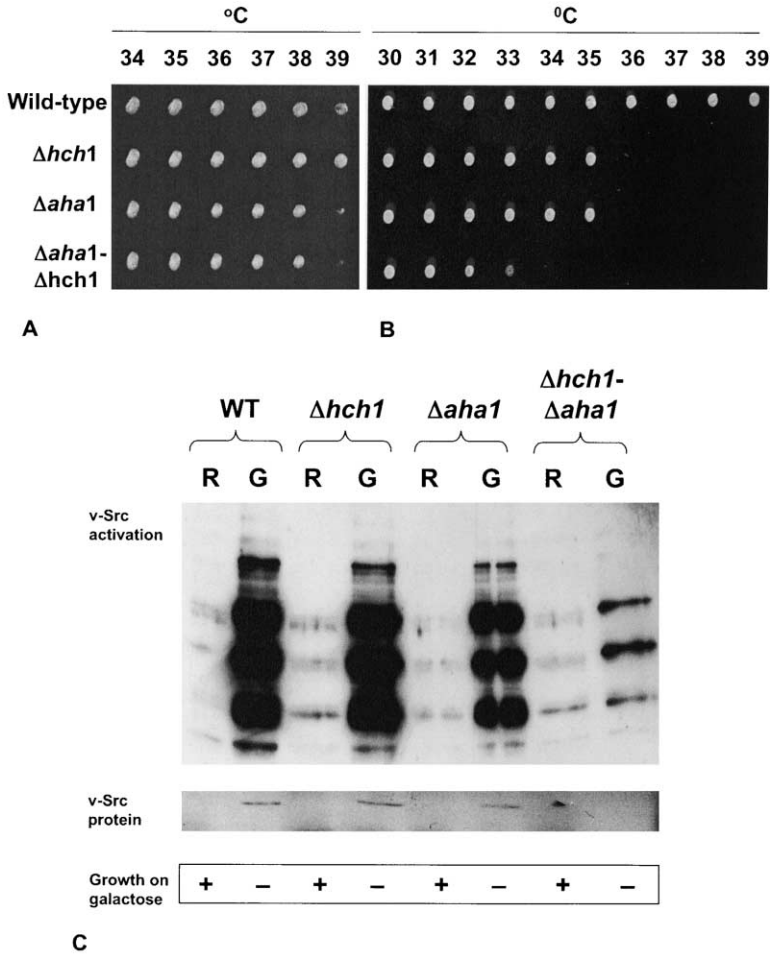

Figure 3. Stress Resistance and v-Src Activation of *AHA1*- and *HCH1*-Deficient Strains

(A) Cells grown on YPDA (30°C × 30 hr) after being subjected to a temperature gradient of 34°C–39°C for 40 hr. Δ*aha1* and Δ*aha1*-Δ*hch1* strains are marginally temperature sensitive (ts).

(B) Cells grown on respiratory medium (YPEG, 30°C) after being subjected to a temperature gradient of 30°C–39°C for 40 hr. Under conditions of increased stress the ts-growth phenotype is more marked, with the double-deleted strain showing a more severe impairment.

(C) Western blot showing protein phosphotyrosine levels in yeast cell extracts following induction of v-Src expression on galactose (G) rather than raffinose (R) medium. Activation of v-Src is compromised in the Δ*aha1* and more severely in Δ*aha1*-Δ*hch1* backgrounds, indicated by lower levels of phosphotyrosine incorporation. The more severe phenotype of the double mutant suggests that Hch1 can to a limited degree restore loss of Aha1 function (see text). Levels of accumulated v-Src are lower in the Δ*aha1* background and almost undetectable in the double delete, consistent with rapid degradation of expressed v-Src when the Hsp90 system is compromised.

growth sensitivity at elevated temperatures (Figure 3A), a phenotype that was enhanced and also shown by the Δ*hch1* strain, during increased stress by growth on respiratory medium (Figure 3B).

To determine whether Aha1 and Hch1 are directly involved in the activation of CPs in vivo, the *v-src* oncogene, encoding a protein tyrosine kinase under the regulation of a galactose promoter, was transformed into *S. cerevisiae* FY 1679-28C and into the Δ*aha1,* Δ*hch1*, and Δ*aha1*-Δ*hch1* mutant strains. Activation of v-Src is strongly dependent on functional interaction with the Hsp90 system in mammalian and avian cells and remains so when expressed in yeast, where its expression is toxic. In all cases, cells were viable on glucose but failed to grow on galactose, indicating that expression of the *v-src* gene was indeed induced on galactose medium and that sufficient active v-Src protein was made to produce toxicity in all the strains (wild-type, Δ*aha1,* Δ*hch1*, and Δ*aha1*-Δ*hch*; data not shown). To investigate the more subtle effects of Aha1 and Hch1 on the activation of v-Src, the cellular phosphotyrosine profile was analyzed immediately after transfer from raffinose to galactose medium. Tyrosine phosphorylation of yeast proteins by v-Src was compromised when the *AHA1* gene was deleted and especially so in the double delete Δ*aha1*-Δ*hch1* background (Figure 3C). This suggests that under these growth conditions it is primarily Aha1 that is required in v-Src activation but can be replaced to some degree by Hch1. The level of accumulated v-Src protein immediately following induction was lower in the Δ*aha1* strain than in wild-type and nearly undetectable in the double delete (Figure 3C). The very low levels of v-Src is not due to loss of expression since cells fail to grow on galactose in common with the wild-type and Δ*aha1,* Δ*hch1* strains. Rather it reflects the destabilization and rapid degradation of unactivated v-Src protein when the Hsp90 system is compromised, as has been previously observed (Nathan et al., 1999). These results indicate that under the growth conditions used, Aha1 and, to a lesser extent, Hch1 are directly involved in Hsp90-dependent v-Src activation.

Hch1 and Aha1 Are Hsp90 Binding Proteins

As the genetic data suggest that Aha1 and/or Hch1 are involved in CP activation, we sought to determine whether Aha1 and Hch1 interact with Hsp90 in vivo, using a selective yeast two-hybrid assay. Neither Aha1 nor Hch1 interacted with themselves or with each other sufficiently strongly to sustain cell growth, even at the lowest level of selection. However, both proteins interacted with Hsp90, with Aha1 able to sustain growth at higher levels of selection (Figure 4A). Furthermore, purified Aha1 incubated with a bacterial lysate containing Flag-tagged Hsp90 could be efficiently immuno-

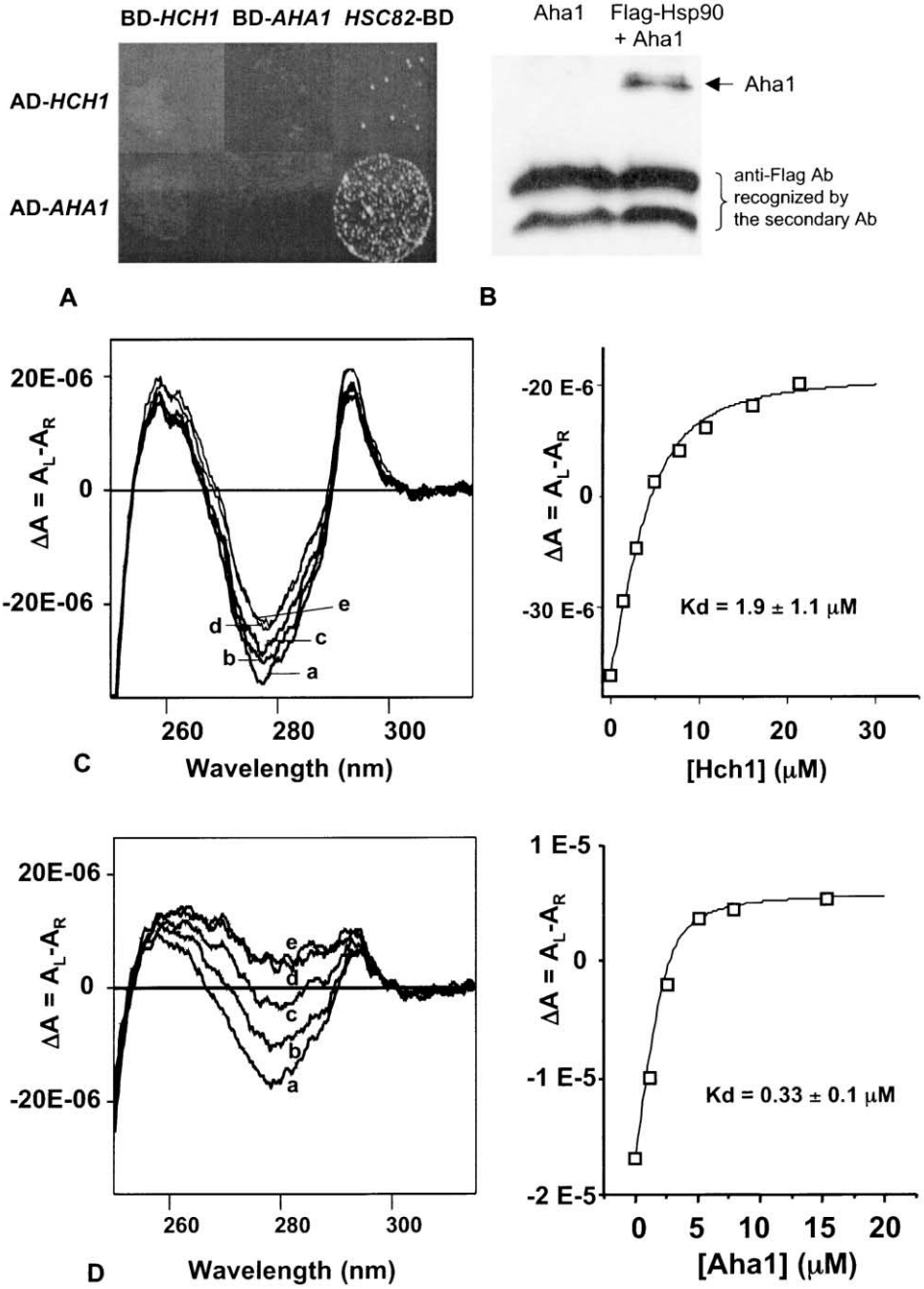

Figure 4. Hch1 and Aha1 Interactions with Hsp90

(A) Growth of colonies in the selective yeast two-hybrid assay indicates interaction between the proteins attached to the DNA binding domain (BD) and transcriptional activation domain (AD) in vivo. Both Aha1 and Hch1 interact with Hsp90 in this assay.

(B) Coimmunoprecipitation (IP) of Flag-tagged Hsp90 and His_6-tagged Aha1 with anti-Flag Ab linked to agarose beads, detected using anti-His_6 Ab. Lane 1, IP of Aha1 without Flag-tagged Hsp90; lane 2, co-IP of Aha1 and Flag-tagged Hsp90. Aha1 co-IPs only in the presence of Flag-tagged Hsp90. Note that the anti-mouse secondary Ab, detecting the primary anti-His_6 Ab, also recognizes the anti-Flag Ab.

(C) Near-UV ΔCD spectra for titration of Hch1 into Hsp90. (a) Hsp90 alone (4.84 μM); (b–e) Hsp90/Hch1 mixtures in the molar ratios of 1:n (n = 0.3, 0.6, 1.0, and 1.5). The K_D was estimated using a Levenberg-Marquardt fit from the binding curve as 1.9 ± 1.1 μM. Saturation of binding was at 1:1.1 (Hsp90:Hch1) indicating that the stoichiometry of the interaction is 1:1.

(D) As in (C) but titration of Aha1 into Hsp90. (a) Hsp90 (2.42 μM) alone; (b–e) Hsp90/Aha1 mixtures in the molar ratios of 1:n (n = 0.48, 1.05, 2.07, and 3.16). The K_D estimated using a Levenberg-Marquardt fit for the interaction was 0.33 ± 0.1 μM, and for Aha1 and Hsp90-AMPPNP it was 0.2 μM (data not shown). Saturation of binding was at 1:1.1 (Hsp90:Aha1) indicating that the stoichiometry of the interaction is 1:1.

precipitated using immobilized anti-Flag Ab, indicating that the interaction of Hsp90 with Aha1 (and by implication Hch1) observed in vivo was direct (Figure 4B). The affinity of Aha1 and Hch1 for Hsp90 was determined using difference circular dichroism (ΔCD) (see Experimental Procedures). Dissociation constants estimated

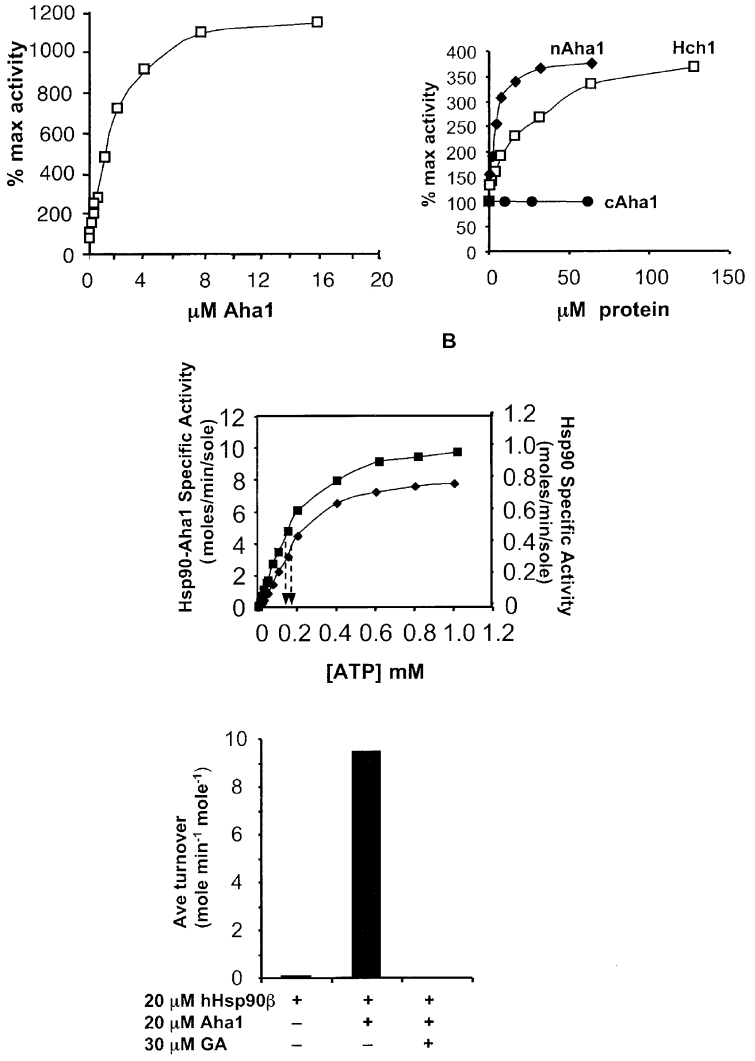

Figure 5. Aha1 Activation of the ATPase Activity of Hsp90 Proteins

(A) Activation of Hsp90 ATPase (2 μM) by Aha1. The amount of Aha1 required for half maximal activation is ≈1.4 μM indicating that a 1:1 stoichiometric complex is formed.

(B) The effect of nAha1 (◆) and Hch1 (□) on the ATPase activity of yeast Hsp90. cAha1 (●) has no effect.

(C) Estimation of the K_M for the ATPase activity of Hsp90 (■ and right-hand scale) and the Hsp90-Aha1 complex (◆ and left-hand scale). The presence of Aha1 does not significantly alter the K_M of the reaction (indicated by arrows).

(D) Activation of human Hsp90β (20 μM) by Aha1 and inhibition by GA.

from the fitted binding curves were 1.9 (±1.1) μM (Hch1) and 0.33 (±0.1) μM (Aha1) with ~1:1 (Hsp90:Aha1/Hch1) stoichiometry in both cases (Figures 4C and 4D).

Aha1 and Hch1 Are Activators of Hsp90's ATPase Activity

The biological activity of Hsp90 depends on its ability to bind and hydrolyze ATP (Panaretou et al., 1998; Obermann et al., 1998), and we have previously shown that this activity is downregulated by the cochaperones Sti1/Hop and Cdc37p/p50^{cdc37} but largely unaffected by others (Prodromou et al., 1999; Siligardi et al., 2002). As Aha1 and Hch1 clearly interact with Hsp90, we sought to determine their effect on the ATPase activity. In initial studies we found that binding of Aha1 and Hch1 to Hsp90 was salt sensitive and a lower-salt buffer was used than in previous studies (see Experimental Procedures). In these assays, Aha1 proved to be a potent activator (Figure 5A), stimulating the ATPase activity of yeast Hsp90 to ~12 times its basal level. Approximately 1.4 μM of Aha1 was required to achieve half maximal activation with 2 μM Hsp90 present in the assay, indicating that a 1:1 stoichiometric complex is formed, consistent with the CD data. Hch1 and the Hch1-homologous N-terminal region of Aha1 (nAha1) also stimulated Hsp90 ATPase but only to ~30% of that achieved by full-length Aha1 (Figure 7B). The C-terminal region of Aha1 in isolation produced no ATPase stimulation at up to a 32 molar excess over Hsp90. The stimulated ATPase activities were fully sensitive to inhibition by the specific Hsp90 ATPase-inhibitor GA, confirming that it was due to hydrolysis of ATP by Hsp90, and not by Aha1 or Hch1, which have no measurable ATPase activity of their own. The near-UV CD-spectrum of isolated Aha1 was unperturbed by the addition of GA, ADP, or AMP-PNP (data not shown) indicating that in isolation Aha1 binds neither nucleotides nor GA. Similarly, the spectrum of Hch1 was unperturbed by the addition of AMP-PNP. To test the possibility that Aha1 may possess a cryptic ATPase activity only manifest when associated with cycling Hsp90, we also determined the effect of saturating levels of Aha1 on the K_D for AMPPNP binding by Hsp90 and on the K_M for the Hsp90 ATPase reaction. The measured K_D of 50 μM (data not shown) compares with previous estimates of 33 μM (Prodromou et al., 2000), and the K_M measurements showed no significant differences

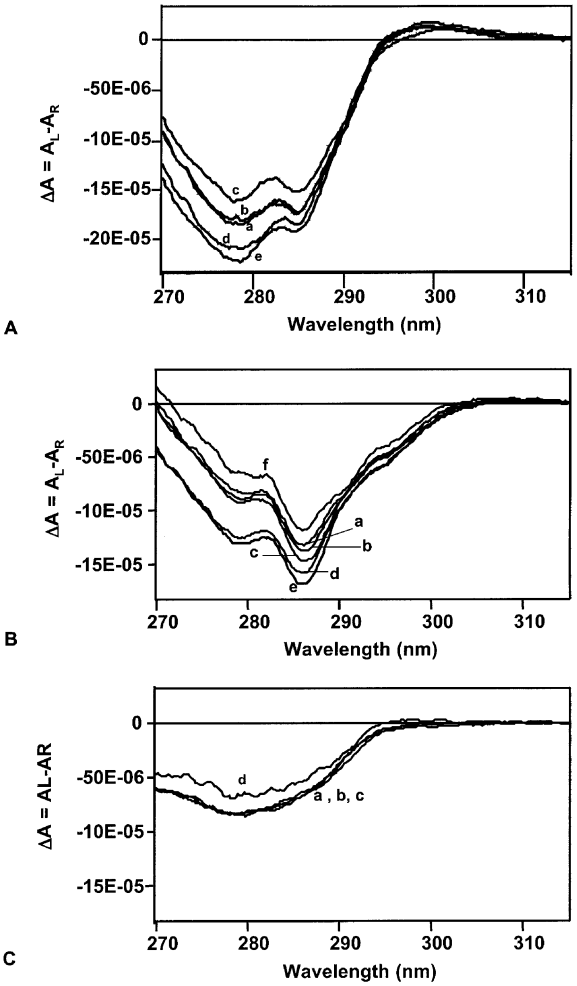

Figure 6. Near-UV Difference-CD Spectra Showing Protein Interactions between Aha1, Hsp90, and Hsp90 Cochaperones

Observed spectra for multicomponent mixtures are compared with calculated spectra for different theoretical complexes, calculated by linear combination of the spectra for the separate components in the same molar ratio as present in the actual mixture.

(A) Near-UV ΔCD for mixtures of Hsp90, Aha1, and cSti1. (a) Observed spectrum for the mixture of Hsp90 (49.6 μM), Aha1 (49.6 μM), and cSti1 (49.6 μM); (b) calculated spectrum for an Aha1-Hsp90-cSti1 complex; (c) calculated spectrum for an Aha1-Hsp90 complex and unbound cSti1; (d) calculated spectrum of an Hsp90-cSti1 complex and unbound Aha1; and (e) calculated spectrum of Hsp90, unbound Aha1, and unbound cSti1. The observed (a) and calculated spectrum of the Aha1-Hsp90-cSti1 complex (b) are essentially identical, indicating that an Aha1-Hsp90-cSti1 complex is formed.

(B) Near-UV ΔCD for mixtures of Hsp90-AMPPNP, Aha1, and Sba1. (a) Observed spectrum for the mixture of Hsp90-AMPPNP (48.82 μM and 97.64 μM, respectively), Aha1 (48.82 μM), and Sba1 (48.82 μM); (b) calculated spectrum for an Aha1-Hsp90-AMPPNP-Sba1 complex; (c) calculated spectrum for an Aha1-Hsp90-AMPPNP complex and unbound Sba1; (d) calculated spectrum of an Hsp90-AMPPNP-Sba1 complex and unbound Aha1; and (e) calculated spectrum of Hsp90-AMPPNP, unbound Aha1, and unbound cSti1. The observed (a) and calculated spectrum of the Aha1-Hsp90-AMPPNP-Sba1 complex (b) are essentially identical, indicating that an Aha1-Hsp90-AMPPNP-Sba1 complex is formed. Furthermore, (f) the near-UV CD spectrum of an Aha1-Hsp90-AMPPNP-Sba1 complex undergoes further structural changes following the addition of Cpr6.

(C) Addition of Cpr6 to an Aha1-Hsp90-AMPPNP-Sba1 complex. Addition of Cpr6 (48.82 μM) to an Aha1-Hsp90-AMPPNP-Sba1 complex (proteins at 48.82 μM and AMPPNP at 97.64 μM) results in changes in the near-UV ΔCD spectrum that are not observed when the complex lacks Aha1. Spectra were calculated by subtracting the observed spectrum for the complex of the components other than Cpr6 from the observed spectrum for that complex with the addition of Cpr6. (a) Observed spectrum of free Cpr6; (b) calculated spectrum of Cpr6 in the presence of Hsp90-AMPPNP complex; (c) calculated spectrum of Cpr6 with AMPPNP, Aha1, and Sba1; and (d) calculated spectrum of Cpr6 in the presence of an Hsp90-AMPPNP-Aha1-Sba1 complex. In all cases the recalculated spectrum for Cpr6 matched the observed spectrum for Cpr6 alone except when Aha1 was present in an Hsp90 complex, suggesting that structural changes occur within the Hsp90 complex when Aha1 and Cpr6 are simultaneously bound. This is consistent with the synergistic effects seen by these cochaperones in ATPase assays.

(Figure 5C). It is reasonable to conclude that the activated ATPase is provided entirely by Hsp90, and results from an increase in K_{cat} produced by binding of Aha1.

In contrast to the yeast protein, the human β-isoform of Hsp90 displays only a very low level of inherent ATPase activity under similar assay conditions. However, addition of Aha1 increased its ATPase activity to readily measurable levels (Figure 5D). In light of its ability to activate the ATPase of Hsp90, we have designated the YDR214W ORF as *AHA1* (activator of Hsp90 ATPase activity).

Aha1 Coexists with Early and Late Hsp90 Cochaperone Complexes

CP activation by Hsp90 proceeds via a series of defined stages characterized in part by the cochaperones present in the Hsp90-CP complex (Smith, 2000). Early complexes are devoid of ATP and contain Sti1/Hop or Cdc37/p50^{cdc37}, which are replaced in later complexes by TPR domain immunophilins such as Cpr6/Cyp40 and FKBP52. Furthermore, the binding of these cochaperones to Hsp90 is mutually exclusive (Owens-Grillo et al., 1996; Silverstein et al., 1998). Sba1/p23 only binds to Hsp90 in the presence of ATP (Fang et al., 1998; Johnson and Toft, 1995) and can coexist with Cpr6 but not Sti1/Hop. Using ΔCD, we analyzed the nature of the complexes that could be formed with Hsp90 by Aha1. Mixtures of Aha1, cSti1, and Hsp90 gave a ΔCD spectrum most consistent with simultaneous binding of Aha1 and cSti1 to Hsp90 (Figure 6A). Similarly, addition of Sba1 and Aha1 to AMPPNP-saturated Hsp90 gave a spectrum consistent with both cochaperones bound simultaneously (Figure 6B). Addition of Cpr6 to this putative Aha1-Hsp90-AMPPNP-Sba1 complex elicited a further spectral change that was not observed when Aha1 was absent from the mixture, suggesting that the interaction of Cpr6 with Hsp90 is altered in the presence of Aha1 (Figure 6C).

Cochaperone Modulation of Aha1-Stimulated Hsp90 ATPase Activity

We have previously shown that the inherent ATPase activity of Hsp90 is inhibited by Sti1/Hop and Cdc37p/p50^{cdc37}, while the TPR-domain immunophilins, such as Cpr6, have no significant direct effect on the ATPase but reactivate it by competitive displacement of Sti1/Hop or Cdc37p/p50^{cdc37} (Prodromou et al., 1999; Siligardi et al., 2002). More recently, we have found that p23/

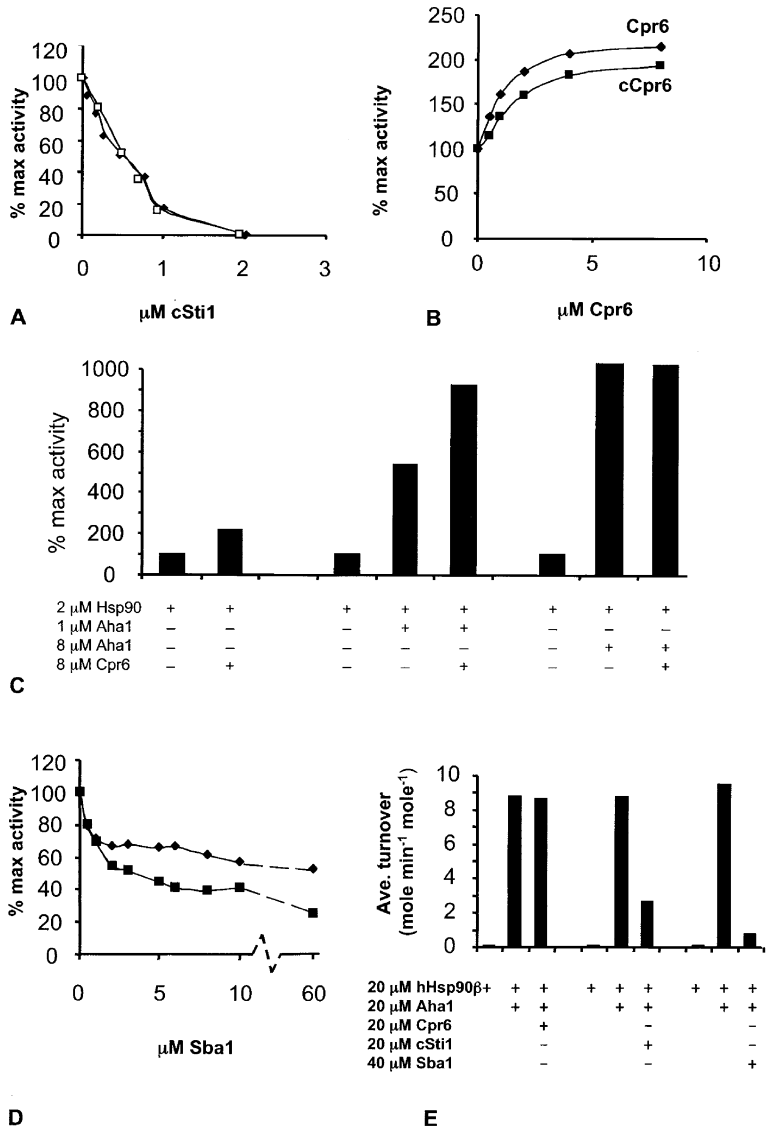

Figure 7. The Effect of Cochaperones on the ATPase Activity of Hsp90 and Its Complexes

Two micromolar of yeast Hsp90 and 1 μM of Aha1 or 20 μM of Hsp90β and 20 μM of Aha1 were used in assays.

(A) Inhibition of Hsp90 (□) and Aha1-Hsp90 (◆) ATPase activities by cSti1. The relative degree of inhibition for a given cSti concentration is very similar.

(B) Activation of the Hsp90 ATPase activity by (◆) Cpr6 and (■) cCpr6. In the high-salt buffer used previously, Cpr6 alone produced no significant ATPase activation.

(C) Synergistic effects of Cpr6 and Aha1. Addition of Cpr6 (8 μM) to Hsp90-Aha1 (2 and 1 μM, respectively) elicits synergistic activation of the ATPase. However, addition of Cpr6 to Aha1-saturated Hsp90 (8 and 2 μM, respectively) has no further effect, indicating that Hsp90 is fully activated by Aha1.

(D) Inhibition by Sba1 of the ATPase activity of (◆) Hsp90 and (■) Aha1-Hsp90 complex. Substantial inhibition of ATPase activity is achieved by moderate concentrations of Sba1; however, further addition of Sba1 does not significantly increase the degree of inhibition, and complete inhibition as observed with Sti1 is not obtained at a 30 molar excess of Sba1 over Hsp90.

(E) Inhibition of the ATPase activity of the hHsp90β-Aha1 complex by Cpr6, cSti1, and Sba1. The synergistic effects of the cochaperones on the Aha1-stimulated ATPase activity of the human Hsp90β complex is the same as that seen for the yeast Hsp90.

Sba1, which binds to the ATP-bound form of Hsp90, also inhibits the inherent ATPase of Hsp90, but more weakly. The K_D for the interaction of Sba1 with AMPPNP-bound Hsp90, estimated by ΔCD spectroscopy, is ~5 μM (data not shown). This relatively low affinity explains earlier observations of a lack of Hsp90 ATPase inhibition by p23 (Young and Hartl, 2000), where the molar ratio of p23 (10 μM) to Hsp90 (5 μM) used would not have been sufficiently high.

As Aha1 appears able to bind Hsp90 in the presence of all cochaperones tested, we investigated the effect of ATPase stimulation by Aha1 on the previously described modulatory activities of these cochaperones. As with free Hsp90, the Aha1-stimulated ATPase activity of Hsp90 was sensitive to Sti1/Hop (Figure 7A), suggesting that the mode of interaction and mechanism of inhibition is the same, regardless of the presence of Aha1. In previous studies, the TPR-domain immunophilin Cpr6 had no significant direct effect on the ATPase of free Hsp90 (Prodromou et al., 1999). However, with the lower-salt buffer utilized here, an ~2-fold increase in basal Hsp90 ATPase activity was observed with either Cpr6 or its C-terminal TPR domain (cCpr6) (Figure 7B), the peptidyl-prolyl isomerase domain (Mayr et al., 2000) being entirely dispensable for this property. Addition of saturating levels of Cpr6 to Hsp90 ~50% saturated with Aha1, nearly doubled the Aha1-stimulated ATPase activity. However, no superstimulation was observed when Cpr6 was added to Hsp90 in the presence of saturating Aha1 (Figure 7C). The activation afforded by the combination of Aha1 and Cpr6 was multiplicative rather than additive, suggesting a synergistic interaction of these proteins when bound to Hsp90, consistent with the additional structural changes observed by CD when Cpr6 and Aha1 are simultaneously present (Figures 6B and 6C).

Sba1 reduced the inherent ATPase activity of Hsp90 and the Aha1-Hsp90 complex (Figure 7D). The degree of inhibition elicited by Sba1 reached a plateau of ~50% at around 1:1 stoichiometry (Sba1:Hsp90), but addition of further Sba1 up to 30 molar excess produced no substantial extra decrease. This behavior is very different from Sti1 or Cdc37, which act early in the chaperone

cycle and can completely inhibit the ATPase activity of Hsp90. Instead, Sba1 acts after the rate-limiting step of ATP binding and consequent dimerization (and possibly hydrolysis) and stabilizes the closed conformation of Hsp90, slowing dissociation of the N-terminal domains and ADP release. Finally, we showed that the effect of the yeast cochaperones on the ATPase activity of the human Hsp90β-Aha1 complex was identical to that of yeast Hsp90, suggesting that these regulatory processes are highly conserved from yeast to humans (Figure 7E).

Discussion

We have identified a family of related proteins, coregulated in yeast and human cells with Hsp90 and its known cochaperones, and involved in stress-tolerance and Hsp90-CP activation in yeast. Together with the demonstration of direct interaction with Hsp90, these data clearly implicate Aha1 and Hch1 as cochaperones of the Hsp90 system.

Aha1 stimulates the inherent ATPase cycle of Hsp90, which is essential for its chaperone activity in vivo (Panaretou et al., 1998; Obermann et al., 1998). Binding and hydrolysis of ATP in the Hsp90 chaperone cycle is directly coupled to changes in the conformational state of the chaperone (Sullivan et al., 1997). Binding of ATP promotes a closed conformation in which the N-terminal domains become physically associated, when then relaxes on ATP-hydrolysis to an open state in which the N-terminal domains are unconstrained (Prodromou et al., 2000; Chadli et al., 2000). Mutational studies indicate that ATP-hydrolysis by Hsp90 is a complex process in which the association of the N-terminal domains in the dimer is rate limiting (Prodromou et al., 2000). As Aha1 does not significantly alter the affinity of Hsp90 for ATP, it does not act as a loading factor and must achieve its activation by promoting the rate-limiting N-terminal dimerization.

Stimulation of ATPase activity by client proteins has been observed in active chaperone systems such as GroEL or Hsp70 (Jackson et al., 1993; Jordan and McMacken, 1995; McCarty et al., 1995; Yifrach and Horovitz, 1996). A similar effect is seen in nonchaperones such as DNA gyrase B, to which Hsp90 is structurally related (Prodromou et al., 1997), where ATPase activity is stimulated by the addition of supercoiled DNA (Kampranis et al., 1999). Stimulation of human Hsp90β ATPase activity by a fragment of the glucorticoid receptor (GR) has also been observed (McLaughlin et al., 2002). However, the GR fragment used in the study was inherently dimeric unlike the true GR client that dimerizes as a result of activation. This unnaturally dimerized GR fragment may stimulate the ATPase by promoting a futile cycle in which Hsp90 is unable to change the conformation of the GR. However, the significance of these observations is unclear as the dimerized GR fragment failed to stimulate the yeast Hsp90 to the same degree, and its ability to bind ligand was not determined.

Direct evidence for the involvement of Aha1 and Hch1 in the activation of v-Src was obtained by analyzing the activation of v-Src immediately after induction, in strains in which Aha1, Hch1, or both were deleted. While Aha1 was of primary importance, a further decrease in v-Src activation in Δ*aha1*-Δ*hch1* strains suggests that Hch1 can replace Aha1 to a limited degree and implicates both proteins in client protein activation by Hsp90. A formal demonstration that the ability of Aha1 (and to a lesser extent Hch1) to stimulate the ATPase activity of Hsp90 in vitro is critical to their involvement in CP activation in vivo will require isolation of Aha1 mutants of wild-type affinity for Hsp90 but with impaired ATPase activation and may not be readily achieved. However, given the complementary involvement of Aha1 and Hch1 in Hsp90 function in vivo and the ability of Aha1, Hch1, and the Hch1-homologous region of Aha1 to activate Hsp90's ATPase activity in vitro, it seems most unlikely that this common in vitro property of Hch1 and Aha1 does not reflect some aspect of their in vivo roles.

The different effects of individual cochaperones on the ATPase activity of Hsp90 reflects the different conformational states of Hsp90 in its chaperone cycle and the specific association of individual cochaperones with particular states and particular classes of client proteins. Compared to previously described cochaperones, Aha1 is unusual in being able to coexist in complexes with early cochaperones such as Hop/Sti1 and p50/Cdc37, and with late cochaperones such as Cyp40/Cpr6 and p23/Sba1. Aha1 may be a general upregulator of Hsp90 function, rather than a stage-specific cochaperone involved in regulation of the lifetime of a particular conformation. As observed when other cochaperones are deleted (Chang et al., 1997; Dolinski et al., 1997; Duina et al., 1996), yeast strains lacking Aha1 and/or Hch1 are viable but are temperature and stress sensitive, and impaired in their ability to activate clients such as v-Src. Thus, the majority of Hsp90 cochaperones (including Aha1) are not essential for the basic operation of the Hsp90 system but may contribute to its efficiency and become particularly important in conditions of elevated stress, where flux through the chaperone cycle my require more stringent regulation. The identification of a specific ATPase activator again emphasizes the key role of the ATPase cycle in Hsp90 function, but how this promotes activation of client proteins, is still to be determined.

Experimental Procedures

Strains and Plasmids

The *HSP83*, *AHA1*, *nAHA1* (residues 1–156), *cAHA1* (residues 157–350), *HCH1*, *cSTI1* (residues 237–589), *CPR6*, *cCPR6* (residues 200–373), *CPR7*, *cCPR7* (residues 202–393), *SBA1*, and the human *HSP90*β genes were cloned into pRSETA as NheI-BamHI, NheI-EcoRI, or NheI-HindIII fragments and expressed as His_6-tagged fusions. The *AHA1* and *HCH1* cloned fragments possessed two nonnative amino acids (LeuGlu) represented by a XhoI at the 3′-OH end of the gene. Genes expressing Flag-tagged Hsp90 and Aha1 (nonnative amino acids, LeuGlu, at its C terminus) were cloned as NdeI-HindIII fragments in pRSETA. The yeast strain FY 1679-28C (*MATa ura3-52, his3* Δ *200, leu2* Δ *1, trp1* Δ *63*) was used to generate the Δ*hch* (*hch1* Δ *kanMX4*), Δ*aha1* (*aha1* Δ *hygB*), and Δ*aha1*-Δ*hch1* (*hch1* Δ *kanMX4, aha1* Δ *hygB*) strains.

Tissue Culture and Stress Regulation of Human *AHA1*

Cells were grown, treated with 50 nM 17AAG or 0.6 μM radicicol, harvested, and lysed as previously described (Hostein et al., 2001) except that antibiotics were omitted. For the heat shock experiment, cells were incubated at 42°C for 30 min, and then returned to 37°C

for 24 hr. Samples (75 μg protein) were separated on a 4%–20% SDS-PAGE gel and then transferred to nitrocellulose membrane. Immunodetection was carried out with either primary antibody (Ab) (rabbit polyclonal Aha1 1:5000 dilution, mouse monoclonal HSP70 SPA810 Stressgen 1:2000 dilution, or mouse monoclonal GAPDH Chemicon 1:2000 dilution) followed by anti-mouse-IgG-HRP or anti-rabbit-IgG-HRP at 1:1000 dilution (Amersham) and finally ECL (Pierce).

RNA Extraction and Microarray Analysis

RNA extraction, labeling (0.5–1 μg of Poly(A)$^+$ mRNA), hybridization conditions, phosphorimaging, and analysis have been previously described (Clarke et al., 2000). Signal intensity was normalized using all data points and a reference mRNA sample comprising mRNA from untreated A2780 ovarian adenocarcinoma cells. Data were organized using self-organizing maps and analyzed as previously described (Eisen et al., 1998).

Protein Extraction and Proteomics

A2780 cells were treated with 60 nM 17AAG (5 × IC_{50}) and harvested after 8, 16, and 24 hr. Control A2780 cells treated with DMSO vehicle alone were harvested at 24 hr. Cell lysate preparations, 2D gel electrophoresis, staining, image analysis (duplicate gels), and spot excision have been previously described (Harris et al., 2002). Protein concentrations were 0.5–1 mg ml^{-1}. Gel spots were prepared and subjected to in-gel digestion with modified trypsin similarly to methods previously described (Clauser et al., 1995). MALDI mass spectrometry was performed on a Reflex III MS in the reflector mode using delayed extraction. The peptide mass fingerprint recorded was internally calibrated using the tryptic autolysis product ions at the monoisotopic masses of m/z 842.5100 and 2211.1046.

Δ*AHA1* and Δ*hch1* Phenotype

The yeast strain FY 1679-28C and mutant strains (Δ*hch1*, Δ*aha1*, and Δ*aha1*-Δ*hch1*) were grown on YPD (2% glucose, 1% yeast extract, and 2% peptone) to midlog phase and diluted in YPD to 1 × 10^4 cells ml^{-1}. One hundred microliter aliquots were inoculated into a 96-well microtiter plate, and a temperature gradient was applied (1°C increment from left [30°C] to right [39°C] per well). After 40 hr growth, cells were replicated to a YPDA plate (YPD + 2% agar) and incubated for 30 hr at 30°C. The same yeast strains were also plated onto synthetic respiratory medium (YPEGA plates, 2% ethanol, 2% glycerol, 6.7%YNB without amino acids, and 2% agar supplemented with appropriate amino acids and nucleotides). Cells were grown at 30°C for 6 days.

Phenotype of vSrc Induction

Cells transformed with the centromeric *ura3* vector, YpRS316v-Src (Murphy et al., 1993; Nathan et al., 1999) expressing v-Src under control of the Gal1 promoter were grown at 30°C on repressive synthetic glucose (SG) medium (2% glucose, 0.67% YNB without amino acids, 2% agar, and supplemented with appropriate amino acids and without uracil) or for induction of v-Src on synthetic galactose (SGa) medium (2% galactose in place of glucose in SG).

Activation and Detection of v-Src-Activity and Protein Levels

Cells transformed with YpRS316v-Src were grown at 30°C to exponential phase in synthetic raffinose (SR) medium (2% raffinose in place of glucose in SG), harvested, and resuspended in 10 ml SR without carbon source. Subsequently, 5 ml aliquots was transferred into SR and SGa, incubated at 30°C for 6 hr, harvested, and then lysed as previously described (Panaretou et al., 1998), except that 0.2 mM sodium orthovanadate was included in the extraction buffer. Lysates fractionated by 12.5% SDS-PAGE were transferred to nitrocellulose membrane. Phosphotyrosine-labeled protein was detected with 4G10 anti-phosphotyrosine Ab (Upstate Biotechnology Incorporated [UBI]), followed by affinity-purified goat anti-mouse HRP-linked Ab (Amersham). Immune complexes were visualized by ECL (Amersham). Similarly, v-Src protein levels were detected with EC10 mouse Ab (UBI) and v-Src activity with 4G10 mouse anti-phosphotyrosine Ab (UBI) followed by secondary goat anti-mouse HRP-linked Ab.

Yeast Two-Hybrid Screening of Protein Interactions

Two-hybrid fusions utilizing the vectors pOAD and pOBD2 were constructed according to published methods (http://depts.washington.edu/sfields/yp_project/YPLM.html) (James et al., 1996; Uetz et al., 2000), and transformants were selected on medium lacking either leucine, for the activator domain (AD) fusions, or tryptophan, for the binding domain (BD) fusions.

A centromeric vector expressing Hsp90-(BD) was constructed in YCplac33 (Gietz and Sugino, 1988) by cloning the 1.3 kb *HSP83* promoter (BamH1-Sal1), the *ADH2* transcription terminator (Pst1-Hind111) from pHSP83b (Panaretou et al., 1998), the *HSP83* coding region (Sal1-Pst1 and lacking the stop codon), and the *GAL4* DNA BD (Pst1 fragment) to yield pBDC90.

AD-ORF and BD-ORF (all except the HSP90-BD) fusions were inserted into the same strain by mating, and diploid cells were selected on medium lacking leucine and tryptophan, which were then screened for protein-protein interactions on the same medium except it was also lacking histidine and supplemented with increasing concentrations (0–4 mM) of 3-amino-1,2,4-triazole. AD-ORF mating to the HSP90-BD fusion was selected on media lacking leucine and uracil and screened for protein-protein interactions on the same medium, except that it was also lacking histidine and supplemented with 0–4 mM 3AT. Colonies were scored after 10 days.

Coimmunoprecipitations

Lysates prepared in buffer A (20 mM Tris [pH 7.5], protease inhibitors [Roche], 0.1% Tween 20, 100 mM NaCl, and 5 mM $MgCl_2$), containing about 10 μg of flag-tagged Hsp90, was mixed with 50 μg of His-tagged Aha1 and incubated for 30 min at 4°C. Subsequently, 50 μl of agarose-conjugated anti-Flag Ab (Sigma) was added, and the lysate was incubated for 60 min at 4°C. The agarose beads were then washed twice at 4°C with buffer A. Proteins retained by the beads were then eluted with with 50 μl SDS-loading buffer, and then 10 μl was loaded onto SDS-PAGE gels for blotting onto nitrocellulose filters. Blots were probed with anti-His_6 mouse Ab (Clontech) followed by anti-mouse HRP-conjugated secondary Ab (Amersham). Localization of the secondary Ab was with the ECL kit (Amersham).

Protein Expression, Purification, and ATPase Assays

Proteins expressed as His_6-tagged fusions were purified as described previously using Talon metal-affinity chromatography, Q-sepharose ion-exchange, and Superdex 75, 200, or sephacryl 400HR gel-filtration chromatography (Panaretou et al., 1998). Proteins were concentrated in 20 mM Tris (pH 7.5) containing 0–25 mM NaCl, 1 mM EDTA, and 0.5 mM DTT. The Hsp90 ATPase assay was performed as previously described (Panaretou et al., 1998), except that the assay conditions were modified (45 mM Tris-Hcl [pH 7.5], 9 mM KCl, and 3 mM $MgCl_2$), and for the nAha1 and Hch1 assays 20 mM Tris-Hcl (pH 7.5), 4 mM KCl, and 1.2 mM $MgCl_2$ was used.

Circular Dichroism Spectroscopy

CD spectra were recorded on a nitrogen-flushed JASCO J720 spectropolarimeter as previously described (Freeman et al., 1998; Prodromou et al., 1999; Siligardi and Hussain, 1998). The molar extinction coefficients used were: $\epsilon_{(Hsp90)}$ = 54050 M^{-1}cm^{-1}, $\epsilon_{(Hch1)}$ = 20940 M^{-1}cm^{-1}, $\epsilon_{(Aha1)}$ = 50160 M^{-1}cm^{-1}, $\epsilon_{(cStit1)}$ = 34480 M^{-1}cm^{-1}, $\epsilon_{(Cpr6)}$ = 18340 M^{-1}cm^{-1}, and $\epsilon_{(Sba1)}$ = 28650 M^{-1}cm^{-1}. A detailed description of the theory and method of difference circular dichroism for investigating protein interactions is given in Siligardi et al. (2002).

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