

Solubility-Promoting Function of Hsp90 Contributes to Client Maturation and Robust Cell Growth

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The Hsp90 chaperone is required for the maturation of signal transduction clients, including many kinases and nuclear steroid hormone receptors. The binding and hydrolysis of ATP by Hsp90 drive conformational rearrangements in three structure domains. Two intrinsically disordered regions of Hsp90 located between these domains and at the C terminus have traditionally been considered to impart flexibility. We discovered that the charged nature of these acid-rich disordered regions imparts a solubility-promoting function to Hsp90 that is important for its cellular activity in yeast. Both the solubility-promoting function and ATPase activity must occur in the same Hsp90 molecule in order to support robust growth, suggesting that the solubility-promoting function is required during the ATP-driven client maturation process. Expression of model clients together with Hsp90 variants indicated interdependent solubilities mediated by the aggregation propensities of both the client and Hsp90. We propose a model whereby the charge-rich disordered regions of Hsp90 serve a solubility-promoting function important for complexes with aggregation-prone clients. These findings demonstrate a novel biological function of the intrinsically disordered regions in Hsp90 and provide a compelling rationale for why their charged properties are conserved throughout eukaryotic evolution.

Chaperones are essential in all organisms, where they perform a number of functions, including promoting the solubility of aggregation-prone proteins. The expression of misfolded proteins capable of aggregating has been shown to decrease fitness in yeast and is correlated with neurodegenerative diseases, including Huntington's disease, amyotrophic lateral sclerosis (ALS), and Alzheimer's disease (8, 15, 16). For these reasons, understanding the role of chaperones in promoting solubility is of central importance to biology and medicine.

The Hsp90 chaperone is required for the maturation of many signal transduction clients, including numerous kinases and steroid hormone receptors (43). Hsp90 clients form signaling pathways that are important in many cancers, and cancer cells can require greater Hsp90 activity than healthy cells (44, 51). These observations have stimulated many efforts to understand the chaperone mechanism of Hsp90 and to develop inhibitors as anticancer therapeutics.

Hsp90 is composed of three domains: the N-terminal (N) domain, which contains an ATPase site; the middle (M) domain; and the C-terminal (C) domain, which forms a thermodynamically stable dimer (1, 33, 35). In addition, Hsp90 has two large regions that are intrinsically disordered: the charged linker (CL) is a region of about 30 amino acids that connects the N and M domains, and the charged extension (CX) is a region of about 25 amino acids at the C terminus. While the structured domains are rigid, large hinge motions between the domains lead to dramatically distinct conformations (1, 40, 41). The two N domains in the Hsp90 homodimer contact one another in the structure of yeast Hsp90 cocrystallized with an ATP analogue and the cochaperone Sba1 (1). In contrast, the N domains are separated by 30 Å in the structure of bacterial Hsp90 cocrystallized with ADP (40). Electron microscopy and fluorescence resonance energy transfer (FRET) experiments have further demonstrated that ATPase-driven conformational changes result in large changes to N-domain distances and that these conformational changes are conserved from bacteria to humans (6, 13, 17, 22, 41). How these

ATPase-driven conformational changes mediate binding with cochaperones and maturation of clients remains an active area of investigation.

The role of the two intrinsically disordered regions of Hsp90 (CL and CX) have been investigated, but their role in antiaggregation function *in vivo* was unclear. The amino acid sequences in these regions are poorly conserved in phylogenetic alignments, yet throughout eukaryotes these regions are consistently rich in negatively charged amino acids (48). These observations suggest that physical properties associated with negative charge, but not the specific sequence of negatively charged amino acids, provides a fitness benefit in eukaryotes. Conformational models of Hsp90 have postulated that the CL and CX may be important for mediating structural rearrangements of the N, M, and C domains (10, 37, 46). In addition, Tsutsumi et al. recently observed that the sequence of the CL can impact *in vivo* function, including the growth rescue of an otherwise lethal N-domain mutation (47). The deletion of unstructured regions of the CL supports yeast growth indistinguishable from that with wild-type Hsp90 across a broad temperature range (10, 21). Deletions that extend into regions ordered within the Hsp90 crystal structure resulted in reduced growth at temperatures of above 37°C (10). While flexibility is clearly inherent to Hsp90 (17, 41), it can be dramatically reduced (10, 34, 46) without impairing yeast growth. In addition, flexibility alone is insufficient to explain the evolutionary conservation of negative charge in the CL.

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Disordered regions of proteins populate multiple conformations. Depending on the amino acid composition and sequence, this conformational heterogeneity can lead to protein association or aggregation (36). For this reason, intrinsically disordered regions are frequently deleted to enable efficient protein purification for *in vitro* biochemical and structural analyses (19). However, the contribution of intrinsically disordered regions to solubility depends on additional biophysical properties, including amino acid composition (23). The high concentration of negative charge in the CL and CX of Hsp90 should interact favorably with aqueous solvent and promote solubility.

Our previous work demonstrated that the CL and CX of Hsp90 promote the solubility of model substrates *in vitro* (48). Both citrate synthase and the nucleotide binding domain of cystic fibrosis transmembrane regulator aggregate at elevated temperature *in vitro*. This aggregation can be dramatically reduced by Hsp90 (14, 48, 54). Individual deletions of the CL or CX resulted in modest decreases in this Hsp90 solubility-promoting function. The double deletion ($\Delta\text{CL}\Delta\text{CX}$) was severely defective for promoting the solubility of these substrates. Of note, these CL and CX deletions were made to remove the bulk of the acid-rich regions without impacting the structured domains of Hsp90. All of the deletion variants were appropriately folded and retained ATPase activity, indicating that the structured domains were not severely perturbed. Importantly, the *in vitro* solubility-promoting function of $\Delta\text{CL}\Delta\text{CX}$ was regained in a charge rescue (CR) variant where an exogenous charge-rich region was appended to the C terminus. These results demonstrated that the intrinsically disordered regions of Hsp90 contribute to solubility promotion with model substrates *in vitro*.

Here we investigated the solubility-promoting role of these intrinsically disordered regions *in vivo*. There are a number of important distinctions between *in vitro* and *in vivo* experiments with Hsp90. Hsp90 is essential in yeast and higher eukaryotes (2, 3). In addition, it is an ATPase, and mutations that abrogate either binding of ATP or hydrolysis do not support viability (29, 33). Thus, the essential function of Hsp90 *in vivo* requires ATP binding and hydrolysis. In contrast, the solubility-promoting activity of Hsp90 observed *in vitro* is not influenced by ATP and is not perturbed by mutations that prevent ATP binding or hydrolysis (48). Experiments with Hsp90 *in vitro*, in the absence of ATP, and with model substrates may not accurately reflect *in vivo* function. To analyze the role of the intrinsically disordered CL and CX *in vivo*, we studied the function of a panel of Hsp90 constructs (wild type, $\Delta\text{CL}\Delta\text{CX}$, and CR) in yeast. Importantly, the CL deletion in these constructs ($\Delta\text{221-252}$) is within a region (positions 217 to 261) that appears to be unstructured in the Hsp90 crystal structure (1). In addition, a larger deletion ($\Delta\text{211-259}$) was shown to support growth similar to that of the wild type across all temperatures (10). Utilizing our unstructured deletion constructs, we observed that the deletion of both the CL and CX impairs growth as well as client solubility and activity.

MATERIALS AND METHODS

Plasmid construction. Wild-type, $\Delta\text{CL}\Delta\text{CX}$ (48), and CR (48) Hsp90 constructs were generated from the yeast *HSP82* gene and cloned into the Trp-marked p414GPD plasmid (25) for expression in yeast. To increase steady-state levels, $\Delta\text{CL}\Delta\text{CX}$ Hsp90 was cloned into the high-copy p424GPD (25) plasmid to generate $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$. A GCN4-based coiled coil computationally optimized for stability (11) was appended to the C

termini of wild-type and D79N Hsp90 to generate constructs that do not heterodimerize with subunits lacking the coil (50). WTcoil and D79Ncoil Hsp90 were cloned into the His-marked p413GPD plasmid (25). All Hsp90 constructs contained a His₆ sequence (GGHHHHHHGGH) at the N terminus to facilitate detection by Western blotting.

Sole Hsp90 in yeast. The haploid *Saccharomyces cerevisiae* strain ECU82a (26) is a derivative of W303 in which both endogenous Hsp90 genes, *HSP82* and *HSC82*, are knocked out and wild-type *HSC82* is constitutively expressed from pKAT6, a *URA3*-marked plasmid that is amenable to negative selection. Lithium acetate was used to introduce Trp-marked Hsp90 constructs into ECU82a, and plating in the absence of tryptophan was used to select for transformants. Transformants were grown in liquid medium lacking tryptophan to cell density of 1×10^7 cells/ml, serially diluted, and plated on synthetic dextrose (SD) medium lacking tryptophan (SD-W medium) at 25°C in the presence or absence of 5-fluoroorotic acid (5-FOA), which selects for loss of the pKAT6 plasmid.

Yeast growth. For monitoring yeast growth at elevated temperature, single colonies of ECU82a strains grown on restreaked plates in the presence of 5-FOA were selected and grown in liquid cultures in SD-W medium to a cell density of 1×10^7 cells/ml. Cultures were serially diluted, plated on SD-W, and grown at the indicated temperatures for 3 to 6 days. To monitor growth in liquid culture, strains were grown for 32 h in SD-W medium at the indicated temperature with dilution to sustain log-phase growth. Cell growth was monitored from 8 to 32 h at the indicated temperature using a Victor X5 multilabel plate reader (Perkin Elmer). The log of relative optical density at 600 nm (OD_{600}) versus time was fit to a linear equation to determine the growth rate.

To measure steady-state levels of the Hsp90 constructs, cultures were grown to a cell density of 1×10^7 cells/ml in SD-W. Cell pellets were lysed by vortexing with glass beads in yeast lysis buffer (50 mM Tris [pH 7.5], 5 mM EDTA, 10 mM phenylmethylsulfonyl fluoride [PMSF]) followed by the addition of sodium dodecyl sulfate (SDS) to 2%. After pelleting of the cellular debris, the protein concentration in the lysates was assessed using the bicinchoninic acid (BCA) assay (Pierce) and the expression level monitored by Western blotting with anti-HisG antibody (Invitrogen).

v-src assay. Lithium acetate was used to introduce p316Galv-srcv5 (49) into ECU82a cells containing wild-type, $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$, or CR Hsp90 constructs as the sole copy of Hsp90. On plates, cells were grown with dextrose as the sugar source to repress *v-src* expression. Liquid cultures were then grown overnight in synthetic raffinose medium lacking uracil and tryptophan (SR-U-W) at 25°C to a cell density of 1×10^7 cells/ml. In order to monitor *v-src* activity in cells completely equilibrated to growth at 36°C, cells were then diluted and grown for 6 h in a shaking incubator at 36°C in SR-U-W to a cell density of 7×10^6 cells/ml. Cell pellets were resuspended and inoculated into medium containing either 2% raffinose (SR-U-W) or 2% galactose (SGal-U-W) as the sugar source. Cells were then grown for an additional 6 h at 36°C. Cell pellets were collected by centrifugation, washed once with water, and frozen at -80°C . Cells were lysed as described previously (49) except that Src lysis buffer contained 10 mM PMSF to inhibit *v-src* degradation. Phosphotyrosine levels were analyzed by Western blotting (3 μg protein/lane) with antibody 4G10 (Upstate). The levels of *v-src* and Hsp90 were analyzed by Western blotting (20 μg protein/lane) with anti-v5 antibody (Invitrogen) and anti-HisG antibody, respectively.

To monitor the degradation of *v-src*, a protein synthesis inhibitor, cycloheximide (CHX) (A.G. Scientific, Inc.) was applied to cells and the time-dependent decrease in *v-src* monitored by Western blotting. Cells were grown as described above except that L-proline was substituted for ammonium sulfate as the sole nitrogen source in the growth medium and 0.003% SDS was added to the SGal-U-W medium to increase the permeability of the yeast cell wall to CHX (20). Cells were grown for 3 h in SGal-U-W, and then CHX was added to 100 $\mu\text{g}/\text{ml}$ and samples were collected after various time in CHX. Cell pellets were collected by centrifugation, washed once with water, and frozen at -80°C . Cells were lysed as described above and *v-src* levels analyzed by Western blotting.

To determine whether galactose-induced expression was impaired in $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ Hsp90 cells, plasmid p316GalSspBv5 (50) was introduced with lithium acetate into ECU82a cells containing wild-type, $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$, or CR Hsp90 constructs as the sole copy of Hsp90. Cells were grown as for *v-src* growth described above with ammonium sulfate as the sole nitrogen source in the growth medium. After allowing cells to equilibrate to growth at 36°C for 6 h in SR-U-W medium to a cell density of 7×10^6 cells/ml, cell pellets were resuspended and inoculated into SGal-U-W medium. Cell pellets were collected by centrifugation at the indicated time points, washed once with water, and frozen at -80°C . Cells were lysed with Src lysis buffer and 10 mM PMSF as described above and the levels of SspB and Hsp90 analyzed by Western blotting.

GR assay. P2A/GRGZ (26) containing a FLAG epitope tag (50) was introduced into ECU82a cells with wild-type, $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$, or CR Hsp90 constructs as the sole copy of Hsp90. Cells were grown overnight in synthetic dextrose medium without adenine and tryptophan (SD-Ade-W) at 25°C to a cell density of 1×10^7 cells/ml. In order to monitor glucocorticoid receptor (GR) activity in cells completely equilibrated to growth at 36°C, cells were then diluted and grown for 8 h in a shaking incubator at 36°C to a cell density of 5×10^6 cells/ml. After 8 h at 36°C, cultures were split in half, and deoxycorticosterone (DOCS) dissolved in ethanol was added to 0 or 10 μM with a final concentration of 0.1% ethanol in all samples. Cells were grown for an additional hour at 36°C and collected by centrifugation. After being washed once with water, cell pellets were frozen at -80°C . Cells were lysed as described previously (49) except that βGal lysis buffer contained 10 mM PMSF to inhibit GR degradation. Galactosidase levels were measured as described previously (49). GR and Hsp90 levels were monitored by Western blotting (20 μg protein/lane) with anti-FLAG antibody (Sigma) for GR and anti-HisG antibody for Hsp90.

The solubilities of Hsp90 and GR were measured in cells grown at 36°C with the addition of 10 μM DOCS as described above. After 9 h at 36°C, cultures were split in half, and cell pellets were collected by centrifugation, washed once with water, and frozen at -80°C . The frozen cell pellets were lysed by vortexing with glass beads in solubility buffer (50 mM Tris [pH 7.5], 5 mM EDTA, 150 mM sodium chloride, 10 mM PMSF, and 1:20 dilution protease inhibitor cocktail [Sigma], with 5% [final concentration] dimethyl sulfoxide [DMSO] used to solubilize the protease inhibitors). For whole-cell lysate samples, SDS was added to 2% immediately after vortexing with glass beads. To isolate soluble fractions, samples were lysed by vortexing with glass beads and insoluble material pelleted by centrifugation. The supernatant representing the soluble protein content of the cell was removed to a fresh tube and SDS added to 2%. The protein concentration in these lysates was assessed using the BCA assay (Pierce). All samples were analyzed by SDS-PAGE with Coomassie blue staining to ensure that loading was appropriately normalized and that lysis was efficient such that endogenous high-molecular-weight proteins were well represented in all soluble fraction samples. The amounts of GR and Hsp90 were measured by Western blotting as described above. The solubility of Hsp90 in the absence of GR was tested under the same conditions in cells that lacked the GR expression plasmid.

RESULTS

The solubility-promoting function of Hsp90 is required for robust yeast growth at elevated temperature. To investigate the solubility-promoting function of Hsp90 activity in unstressed cells, we compared the abilities of wild-type, $\Delta\text{CL}\Delta\text{CX}$, and CR Hsp90 (Fig. 1A) to support yeast viability as the sole copy of Hsp90 at modest temperature. Each construct was introduced into a yeast strain whose only other source of Hsp90 was encoded on a *URA3* plasmid that could be swapped out using 5-FOA (26, 49). $\Delta\text{CL}\Delta\text{CX}$ and CR Hsp90 both supported yeast growth as the sole Hsp90 in yeast (Fig. 1B), and both constructs accumulated to wild-type levels in yeast grown at 25°C (Fig. 1C).

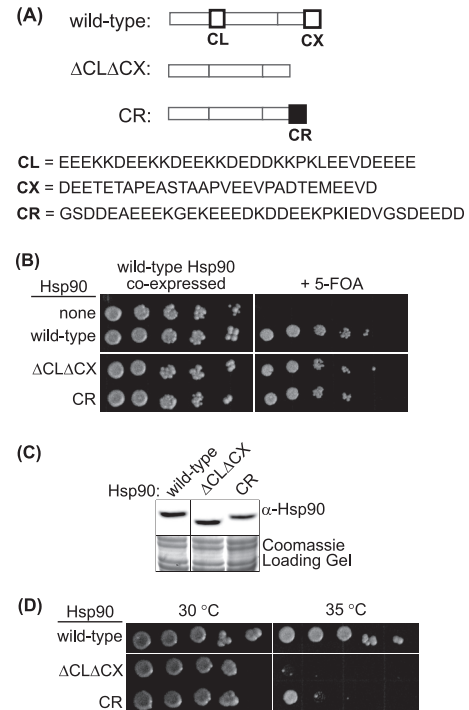


FIG 1 The solubility-promoting function of Hsp90 is required for yeast growth at elevated temperatures. (A) Hsp90 constructs used in these studies. Primary structures of wild-type $\Delta\text{CL}\Delta\text{CX}$, and CR Hsp90 are shown. Deletions in $\Delta\text{CL}\Delta\text{CX}$ were limited to unstructured regions. The CR sequence is from the CL region of human Hsp90. (B) $\Delta\text{CL}\Delta\text{CX}$ and CR Hsp90 supported yeast viability as the sole copy of Hsp90 at 25°C. (C) $\Delta\text{CL}\Delta\text{CX}$ and CR Hsp90 accumulated to wild-type levels at 25°C based on Western blotting of yeast lysates. Intervening lanes were removed for presentation purposes. (D) Growth of yeast strains expressing wild-type, $\Delta\text{CL}\Delta\text{CX}$, or CR Hsp90 as the sole copy of Hsp90 at 30 and 35°C.

We conjectured that yeast harboring $\Delta\text{CL}\Delta\text{CX}$ Hsp90 may exhibit growth defects at elevated temperature, where robust growth shows an increased dependence on Hsp90 levels (3) that has been linked to cell wall integrity (CWI) pathways (12, 39, 45). We examined growth at elevated temperatures (Fig. 1D) and observed that $\Delta\text{CL}\Delta\text{CX}$ Hsp90 was deficient for growth at 35°C compared to wild-type Hsp90. CR Hsp90, which displays efficient solubility-promoting activity *in vitro* (48), was less severely impaired for growth at elevated temperature than $\Delta\text{CL}\Delta\text{CX}$ Hsp90 (Fig. 1D).

To accurately quantify growth defects, we examined the growth properties of wild-type, $\Delta\text{CL}\Delta\text{CX}$, and CR Hsp90 in liquid culture at elevated temperature (Fig. 2A). At 37°C we observed that $\Delta\text{CL}\Delta\text{CX}$ Hsp90 had essentially stopped growing compared to cells with wild-type Hsp90 and that CR Hsp90 recovered about 60% of the growth rate of the wild type. Culture temperatures were monitored using a thermocoupler submerged in a mock culture and were found to vary by $\pm 0.2^\circ\text{C}$ around the set temperature. Of note, similar analyses of temperatures on plates indicated a wider temperature variation. At 37°C in liquid culture, the expression level of $\Delta\text{CL}\Delta\text{CX}$ Hsp90 is clearly reduced relative to those of both wild-type and CR Hsp90 (Fig. 2B). In order to distinguish expression-level effects (3) from other biochemical functions such as client solubility, we increased expression of $\Delta\text{CL}\Delta\text{CX}$ Hsp90 by generating a high-copy expression plasmid ($\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$). $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ Hsp90 was expressed to similar levels

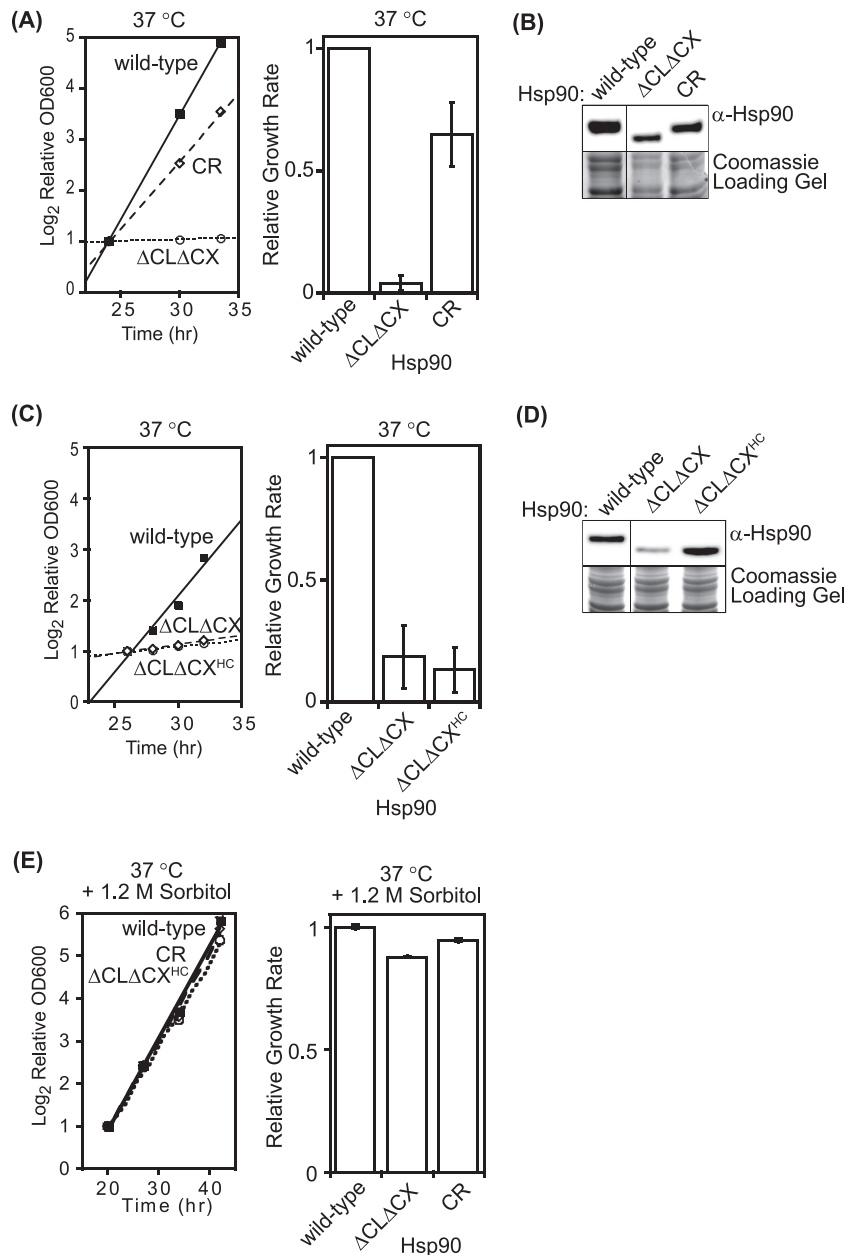


FIG 2 Quantification of growth defects of $\Delta\text{CL}\Delta\text{CX}$ Hsp90. (A) Growth of $\Delta\text{CL}\Delta\text{CX}$ Hsp90 in liquid culture was significantly impaired at 37°C. Experiments were performed in triplicate (on the left are results from a representative experiment) and analyzed to determine the average growth effect and the variation between replicates. (B) The steady-state level of $\Delta\text{CL}\Delta\text{CX}$ Hsp90 was reduced compared to that of wild-type Hsp90 at 37°C based on Western blotting of yeast lysates. Intervening lanes were removed for presentation purposes. (C) In order to increase $\Delta\text{CL}\Delta\text{CX}$ Hsp90 expression, we generated a high-copy expression plasmid ($\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$). $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ Hsp90 was defective for growth in liquid culture at 37°C. Experiments were performed in triplicate (on the left are results from a representative experiment) and analyzed to determine the average growth effect and the variation between replicates. (D) $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ Hsp90 accumulated to wild-type levels at 37°C as determined by Western blotting of yeast lysates. Intervening lanes were removed for presentation purposes. (E) Growth rates at 37°C with 1.2 M sorbitol. For growth measurements, error bars represent standard deviations ($n = 3$).

as wild-type Hsp90 but did not improve the growth rate of yeast at 37°C (Fig. 2C and D). Of note, the C-terminal peptide (MEEVD) of wild-type Hsp90 binds to cochaperones, and this region is deleted in both $\Delta\text{CL}\Delta\text{CX}$ and CR Hsp90. Individual deletion of the MEEVD region from Hsp90 has been reported and was not observed to compromise yeast viability (21). The absence of the MEEVD motif from CR Hsp90 together with our observation that CR strongly rescues the growth defect of $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ (Fig. 2A)

indicates that the dominant functional effect of deleting the CX is not due to the MEEVD sequence. Instead, our results indicate that the charged property of Hsp90 is essential for growth at elevated temperature independent of expression level. The improved growth of the CR construct compared to $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ indicates that the charged nature of the unstructured regions is a primary contributor to growth rate under these conditions.

Robust growth of yeast at elevated temperature requires

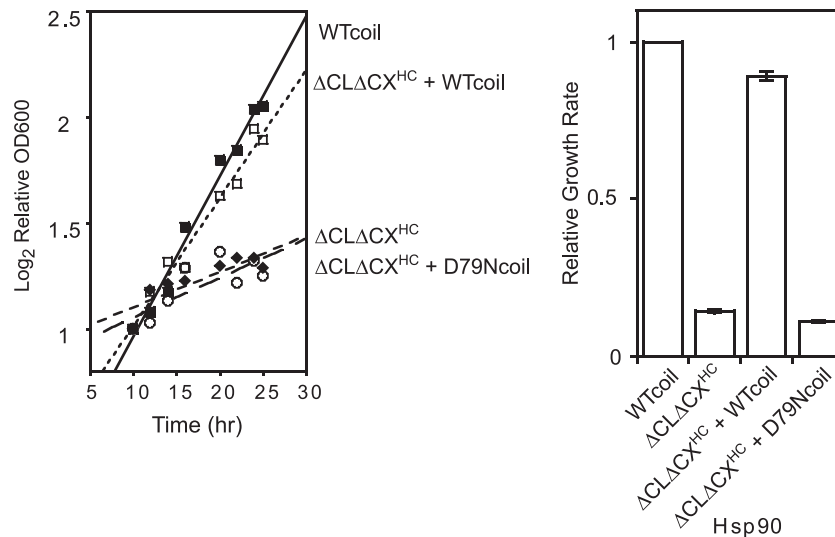


FIG 3 Solubility-promoting and ATPase activities were required in the same Hsp90 molecule for robust growth. The growth deficiency of $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ was not dominant and could be rescued by coexpression of a functional WTcoil Hsp90. In contrast, coexpression of $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ with an ATP-deficient D79Ncoil failed to support robust growth. Experiments were performed in triplicate (on the left are results from a representative experiment) and analyzed to determine the average growth effect and the variation between replicates. Experiments were performed at 37°C and error bars represent standard deviations ($n = 3$). Coiled-coil constructs were used to prevent cross-dimerization.

Hsp90-mediated activation of the cell wall integrity (CWI) signaling pathway (18, 45). To examine potential CWI defects in yeast harboring our Hsp90 constructs, we examined growth in the presence of the osmotic stabilizer sorbitol, which was previously shown to enable robust yeast growth in the presence of CWI signaling defects (45). We observed that 1.2 M sorbitol almost fully rescued the growth defects of $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ Hsp90 at 37°C (Fig. 2E). These results suggest that CWI signaling is compromised by $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ Hsp90.

Coexpression of $\Delta\text{CL}\Delta\text{CX}$ with wild-type Hsp90. To gain mechanistic insights into the deficiency of $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$, we performed coexpression experiments. Because Hsp90 is a dimer, coexpression experiments can be complicated to analyze due to the formation of heterodimer Hsp90 species. To prevent cross dimerization in coexpression experiments, we utilize a coiled coil on one construct. The coiled coil results in superstabilization of one homodimer, which shifts the dimer exchange reaction to disfavor any heterodimer formation (50). Using this strategy, we observed that coexpression of WTcoil Hsp90 with $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ largely rescued the growth defect of $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ (Fig. 3). Of note, the coiled coil caused a slow-growth phenotype at 37°C compared to that of wild-type Hsp90, suggesting that transient C-domain dissociation may be important for robust yeast growth at elevated temperatures. For this reason, it was important in these experiments to compare rescue (WTcoil Hsp90 with $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$) to yeast with WTcoil as the sole Hsp90. This comparison indicates that the primary effect of $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ at elevated temperatures is a loss of function.

Both the ATPase function and the solubility-promoting function are important for robust yeast growth. We examined whether these functions were required in the same Hsp90 molecule or if a solubility-promoting mutant ($\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$) coexpressed with an ATPase mutant (D79Ncoil) would support robust growth. D79Ncoil Hsp90 contains a point mutation that abolishes ATP binding and does not support viability on its own (27, 28, 50) but

retains the ability to function as a solubility-promoting chaperone *in vitro* (48). D79Ncoil also includes an engineered coiled coil to prevent cross dimerization (50) with $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$. D79Ncoil coexpression failed to rescue the growth defect of $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ Hsp90 at 37°C (Fig. 3). This result indicates that both the solubility-promoting function and ATPase function of Hsp90 must occur in the same molecule, likely because the solubility-promoting function is required during the ATP-driven client maturation process.

In order to facilitate examination of the ATP-dependent activation of clients, we examined the sensitivity of our Hsp90 panel to small changes in temperature by monitoring growth at 36°C. At this temperature, the rank order of growth rates for wild-type, $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$, and CR Hsp90 is the same as at 37°C, but the magnitude of growth defects is reduced about 2-fold (see Fig. S1 in the supplemental material). Of note, large changes in growth rates are frequently observed for small temperature changes close to the critical temperature required to stall growth (26). The ability of all Hsp90 variants to grow at this temperature enabled further biochemical analysis of client proteins under these conditions. Because the growth was sensitive to small changes in temperature, care was taken to perform experiments on all strains in parallel and the culture temperature was monitored periodically using a sterilized thermocoupler.

$\Delta\text{CL}\Delta\text{CX}$ is deficient for client expression and activation at elevated temperature. We investigated the ability of $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ to mature the *v-src* tyrosine kinase, which has a well-established dependence on Hsp90 for activity in yeast (5, 26, 53). Yeasts have very low levels of phosphotyrosine, and *v-src* activity can be readily observed by following tyrosine phosphorylation of endogenous yeast proteins by active *v-src*. We observed that in $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ yeast, *v-src* accumulated at low levels and did not exhibit *src* kinase activity (Fig. 4A). In contrast, CR Hsp90 supported *v-src* expression and kinase activity indistinguishably from wild-type Hsp90.

We examined the possibility that the poor expression of *v-src*

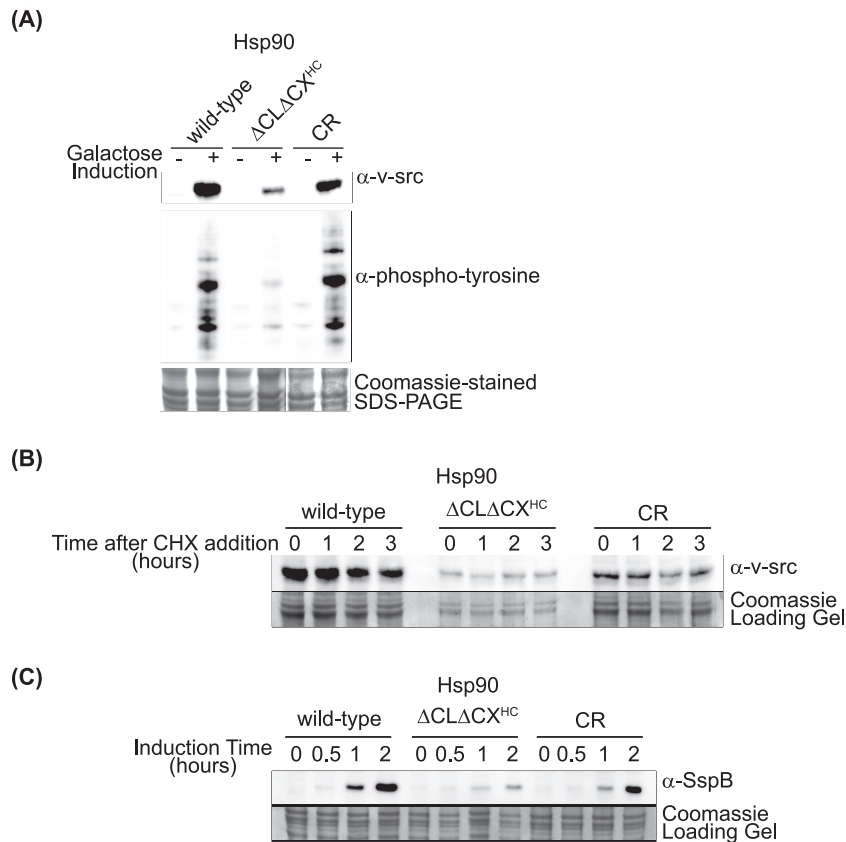


FIG 4 Yeast with Δ CL Δ CX^{HC} Hsp90 was deficient for galactose-induced expression and activity of *v-src* kinase. (A) Yeast with Δ CL Δ CX^{HC} Hsp90 had reduced *v-src* activity as judged by phosphotyrosine buildup and had reduced *v-src* levels. CR Hsp90 activated *v-src* to wild-type levels. Intervening lanes were removed for presentation purposes. (B) Degradation of *v-src* over time was monitored following the addition of the protein synthesis inhibitor CHX by Western blotting for *v-src* in yeast lysates. (C) Galactose-induced expression was impaired in yeast with Δ CL Δ CX^{HC} Hsp90 as monitored with the non-Hsp90 client SspB.

with Δ CL Δ CX^{HC} was due to rapid *v-src* degradation by utilizing cycloheximide to block new protein synthesis and following *v-src* levels as a function of time (Fig. 4B). We found that *v-src* levels declined slowly and similarly over a 3-h time course for Δ CL Δ CX^{HC}, wild-type, and CR Hsp90. These results indicate that variations in the *v-src* degradation rate between the strains were modest and suggested that reduced *v-src* levels may be caused instead by reduced induction from the galactose-inducible promoter used to drive *v-src* expression.

We probed whether Δ CL Δ CX^{HC} Hsp90 was deficient for expression from the galactose-inducible promoter by using the same promoter to drive the expression of SspB, a non-Hsp90-dependent protein that is highly soluble (50). We observed that galactose-induced expression of SspB is reduced and/or delayed in cells expressing Δ CL Δ CX^{HC} Hsp90 compared to wild-type Hsp90 (Fig. 4C). Previous studies have shown that Hsp90 activity is required for efficient transcriptional activation from the galactose promoter (7). Our results implicate the charge-rich regions of Hsp90 in this process, though this hindered our ability to investigate activation of *v-src*.

In order to analyze an Hsp90 client that does not require induced client expression, we turned to the glucocorticoid receptor (GR). Like *v-src*, GR is a well established Hsp90-dependent client in yeast (31), but unlike *v-src*, it can be constitutively expressed. Upon stimulation with agonist, GR transcriptional activity in

yeast was monitored with a β -galactosidase reporter. At elevated temperature, we observed that cells with either Δ CL Δ CX^{HC} or CR Hsp90 were deficient for GR activity compared to cells with wild-type Hsp90 (Fig. 5A). Consistent with our observations, previous reports have demonstrated that the sequence of the CL can affect GR maturation in yeast (47). In the absence of agonist, all samples had baseline β -galactosidase levels that were less than 10% of those in wild-type Hsp90 samples with agonist (data not shown). The total levels of GR in cells with Δ CL Δ CX^{HC}, CR, and wild-type Hsp90 were similar (Fig. 5B), indicating that Δ CL Δ CX^{HC} and CR Hsp90 are deficient at maturing GR to an active state and not in maintaining the expression level.

Motivated by previous observations that the GR protein is poorly soluble in its purified form (38), we investigated the solubility of GR in cells. In cells with wild-type Hsp90, GR was abundant in the soluble fraction, while in cells with either Δ CL Δ CX^{HC} or CR Hsp90, the soluble fraction contained very low levels of GR (Fig. 5B). Thus, low levels of GR in the soluble fraction correspond to reduced GR-driven reporter activity. These results suggest that Hsp90 assists GR maturation by affecting the solubility of GR and that the CL and CX regions of Hsp90 are required for this solubility.

While all of the Hsp90 constructs in this study are readily soluble in purified form (48), we considered the possibility that they may be partly insoluble under cellular conditions, where Hsp90 is

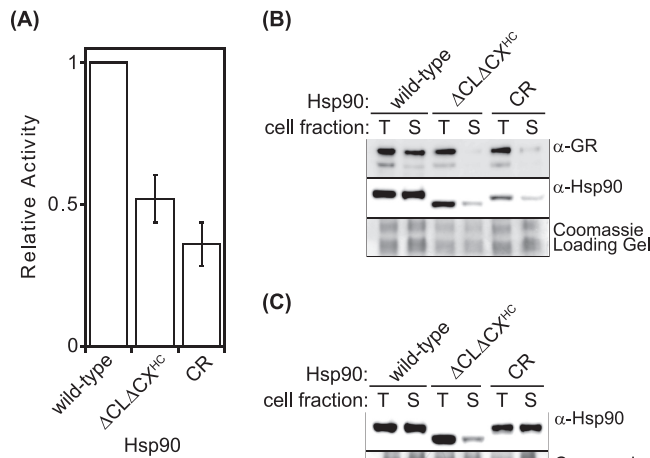


FIG 5 $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ Hsp90 was unable to mature the hormone receptor GR in yeast. (A) GR reporter activity levels were lower than those of the wild type for both $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ and CR Hsp90. Error bars represent standard deviations ($n = 3$). (B) Western blotting for GR in whole-cell lysates (T) and soluble fractions (S) indicated that the ratio of soluble to total GR is drastically reduced in $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ and CR Hsp90 cells. The solubilities of $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ and CR Hsp90 were also reduced compared to that of wild-type Hsp90. (C) In the absence of exogenous client proteins, the solubility of $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ Hsp90 was drastically reduced, while CR Hsp90 maintained wild-type solubility.

one of the most concentrated proteins in the cytosol (3) and binds to hundreds of partner proteins (55). We had previously observed that $\Delta\text{CL}\Delta\text{CX}$ is indistinguishable from wild-type Hsp90 for aggregation at 45°C but that in the presence of a purified aggregation-prone client $\Delta\text{CL}\Delta\text{CX}$ was dramatically less stable than wild-type Hsp90 (48). Motivated by these *in vitro* observations, we examined the impact of exogenous expression of the aggregation-prone client, GR, in yeast. When GR was expressed, we observed that both $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ and CR Hsp90 accumulated at reduced levels in soluble fractions compared to wild-type Hsp90 (Fig. 5B). We further examined Hsp90 solubility in cells without GR and found that $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ Hsp90 accumulated at reduced levels in soluble fractions but that CR Hsp90 had solubility properties similar to those of wild-type Hsp90 (Fig. 5C). These results indicate that the charge-rich CL and CX regions of Hsp90 can have a large impact on the solubility and activity of Hsp90 in cells with endogenous clients and that this can be magnified by exogenous expression of the aggregation-prone GR client.

DISCUSSION

The results presented here indicate that the charge-rich regions of Hsp90 (CL and CX) contribute solubility-promoting properties to Hsp90 and Hsp90-client complexes in cells. We propose that the solubility-promoting properties of these charge-rich regions serves to improve the solubility of Hsp90 itself and complexes of Hsp90 with aggregation-prone clients (Fig. 6). Many Hsp90 client proteins are aggregation prone, particularly steroid hormone receptors such as GR (30, 32). Our results suggest that Hsp90 is required for maintaining the balance between soluble, active clients and insoluble, inactive proteins (Fig. 6). Our results indicate that the net charge of Hsp90 contributed by its two charge-rich regions (CL and CX) is important for maintaining this balance.

Based on our results and those of others (47), the solubility-

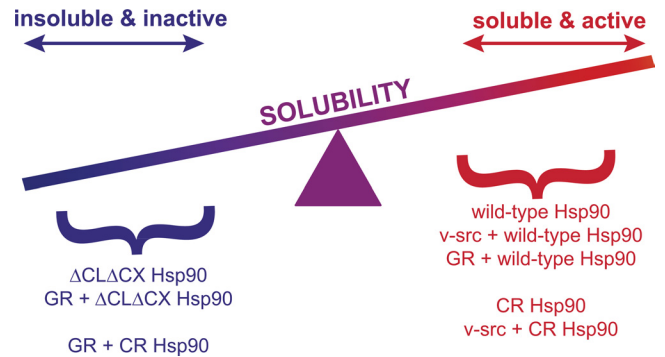


FIG 6 Hsp90 plays an important role in maintaining the balance between soluble, active clients and insoluble, inactive proteins. The solubilities and/or activities of Hsp90 variants and clients monitored in this study are outlined. The high charge of Hsp90 concentrated in two charge-rich regions modulates this solubility-promoting function. Deletion of both charge-rich regions of Hsp90 impairs the solubility of Hsp90 and in turn reduces the solubility of complexes with clients.

promoting contribution of charge-rich regions likely depends on their structural location, net charge, and sequence. For example, we observed that Hsp90 lacking both charge-rich regions ($\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$) is aggregation prone on its own in yeast but that both wild-type Hsp90 and CR Hsp90, where one of these regions is replaced with an exogenous charge-rich sequence, are highly soluble. These results indicate that having a single charge-rich sequence is sufficient to imbue Hsp90 in yeast cells with high solubility. In contrast, when an aggregation-prone client (GR) was exogenously expressed, we observed that only wild-type Hsp90 maintained it in a soluble form and that both CR and $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ Hsp90 became aggregation prone. In our model (Fig. 6), the aggregation properties of GR require the solubility-promoting function of both CL and CX in order to confer net solubility on Hsp90-GR complexes.

The solubility-promoting effects of the CL and CX regions may be particularly important during the process of client maturation. Studies using chemical modification of residues in the hormone binding domain of GR suggest that Hsp90 is required for opening the steroid binding site of GR, making it accessible for hormone binding (42). Opening of the hormone binding domain results in exposure of the hydrophobic binding pocket (9, 24, 42, 52), which likely causes a transient state that is highly prone to aggregate. The solubility-promoting function of Hsp90 may play a role in enhancing the solubility of GR in this hormone binding-competent conformation.

The negatively charged properties of the Hsp90 CL and CX regions are maintained throughout the eukaryotic evolutionary lineage (21, 48), implying that they contribute to the function of Hsp90 in these organisms. The CX is at the C terminus of Hsp90 and contains a peptide motif that binds to tetratricopeptide repeat domain (TPR)-containing cochaperones (4). However, binding to these cochaperones does not appear to be critical to function in yeast, as deletion of the CX is compatible with yeast viability and efficient maturation of nuclear steroid receptors in yeast (21). The CL connects the N and M domains, which both contribute catalytic amino acids for ATP hydrolysis. The CL has been implicated in the ATP- and cochaperone-driven conformational cycle of Hsp90 (10, 22, 34, 41). Our results indicate an additional critical

function of CL and CX: imparting high solubility on Hsp90 in complex with aggregation-prone clients.

Multiple lines of evidence and reasoning indicate that the charge-mediated Hsp90 solubility imparted by the disordered CL and CX regions should be advantageous. The expression of aggregation-prone proteins results in an inefficient use of cellular resources in terms of both wasted biosynthetic energy and additional energy resources required to rescue or degrade proteins in aggregates. The fitness impact due to expression of aggregation-prone proteins can be magnified because initial aggregates can seed the aggregation of other proteins. The fitness cost caused by the expression of a misfolded protein has been directly observed in yeast (8) and indicates that solubility is under selective pressure. Because the fitness cost of an aggregation-prone protein should be related to its expression level, the solubility of Hsp90, which is one of the most highly expressed proteins, should be under strong selective pressure. This conceptual framework combined with our observations of the solubility-promoting properties of the charge-rich regions provides a compelling explanation for conservation of the charged properties of Hsp90 CL and CX throughout eukaryotic phylogeny.

Future directions. Chaperones are critical to all cells because of the inherent challenges of protein folding in the complex and crowded cellular environment. However, mechanistic investigations of chaperones in cells have been hindered because of overlapping/redundant chaperone function. The unique specificity of Hsp90 clients combined with the ability to modulate the solubility-promoting function of Hsp90 through deletions of the charge-rich regions provides a promising system to study aggregation events in cells. Our results indicate that Hsp90 is involved in maintaining the balance between soluble, active clients and insoluble, inactive proteins. Solubility-deficient $\Delta\text{CL}\Delta\text{CX}^{\text{HC}}$ Hsp90 provides a novel tool for identifying endogenous aggregation-prone clients of Hsp90 in cells. This should be a promising route to enhance our understanding of the role of chaperones such as Hsp90 in the solubility of cellular proteins.

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