Published in final edited form as:

J Mol Biol. 2010 September 3; 401(5): 931–939. doi:10.1016/j.jmb.2010.06.066.

Charge-rich regions modulate the anti-aggregation activity of Hsp90

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Abstract

Protein aggregation can have dramatic effects on cellular function and plays a causative role in many human diseases. In all cells, molecular chaperones bind to aggregation-prone proteins and hinder aggregation. The ability of a protein to resist aggregation and remain soluble in aqueous solution is linked to the physical properties of the protein. Numerous physical studies demonstrate that charged atoms favor solubility. We note that many molecular chaperones possess a substantial negative charge that may allow them to impart solubility on aggregation-prone proteins. Hsp90 is one such negatively charged molecular chaperone. The charge on Hsp90 is largely concentrated in two highly acidic regions. To investigate the relationship between chaperone charge and protein solubility, these charge-rich regions were deleted and the resulting Hsp90 constructs analyzed for anti-aggregation activity. We found that deletion of both charge-rich regions dramatically impaired Hsp90 anti-aggregation activity. The anti-aggregation role of the deleted charge-rich regions could be due to net charge or sequence-specific features. To distinguish these possibilities, we attached an acid-rich region with a distinct amino acid sequence to our double-deleted Hsp90 construct. This charge-rescue construct displayed effective anti-aggregation activity indicating that the net charge of Hsp90 contributes to its anti-aggregation activity.

Keywords

protein; mis-folding; aggregation; chaperone; charge; Hsp90

Introduction

The propensity of a protein to aggregate is linked to the physical properties of the protein. Understanding these physical properties has important implications for many biological processes that involve protein aggregation including numerous human diseases.^{1–6} The propensity of a protein to aggregate is governed by the energetic and kinetic balance between interactions of the protein with solvent versus self-association. Both of these interactions must be considered in order to understand the process of protein aggregation.

The strength of protein self-association is dramatically influenced by hydrophobic contacts.⁷ By chemical definition, hydrophobic groups prefer to associate with other hydrophobic groups relative to aqueous solvent. In order to remain soluble, most natively-folded

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cytoplasmic proteins sequester hydrophobic side-chains from solvent in the core of the protein. These hydrophobic side-chains become increasingly exposed to solvent when proteins transiently sample mis-folded conformations. For this reason, mis-folded conformations frequently aggregate in an energetic effort to bury exposed hydrophobic surfaces.⁸

In contrast to hydrophobic effects, charged amino acids have been shown to increase protein solubility. It is well appreciated in biochemistry that proteins are usually least soluble at a pH where their net charge is zero.⁹ From a chemical perspective, charged groups interact favorably with aqueous solvent and are unlikely to self-associate. Coulombic repulsive forces between like-charges also influence self-association. Experimentally increasing the net charge of aggregation-prone proteins, and increasing Coulombic self-repulsion, has been shown to improve protein solubility.^{10,11}

Because aggregation is a concentration-dependent event, the aggregation of mis-folded proteins is enhanced by the crowded environment of the cell.^{3,8,12,13} In cells, many aggregation-prone proteins rely on interactions with molecular chaperones.^{3,4,8,12-14} Molecular chaperones hinder protein aggregation and facilitate folding and assembly of proteins into higher order structures.¹⁴⁻¹⁶ Many molecular chaperones bind to mis-folded proteins and reduce aggregation by shielding hydrophobic side-chains from bulk solvent.⁸ For example, the GroEL (Hsp60) chaperone forms a closed barrel around mis-folded proteins, effectively shielding the entire protein from bulk solvent.^{17,18}

Other chaperones, including Hsp90 and Hsp70, have smaller hydrophobic patches capable of binding to mis-folded proteins and partially shielding exposed hydrophobic side-chains from solvent.^{19,20} Because these systems do not form closed barrels, they can not completely shield mis-folded proteins from solvent exposure. The solubility of this type of complex may benefit from additional pro-solubility mechanisms. A high net charge on the chaperone complex is one physical property that may improve solubility. The net charge on a chaperone complex has the potential to influence solubility through direct solvent interactions and Coulombic self-repulsion.

Results

Negative charge is a common feature of molecular chaperones

We observed that three chaperones with anti-aggregation activity (Hsp90, Hsp70, and Hsp33) are characterized by a high negative charge that is evolutionarily conserved (Table 1). These three highly charged chaperones all have hydrophobic patches that are could partially shield mis-folded proteins from solvent.¹⁹⁻²¹ In addition, the Hsp60 chaperone completely encloses mis-folded proteins¹⁷ and is not highly charged. For comparison, the non-chaperone protein triosphosphate isomerase is also not highly charged. These observations suggest that the conservation of negative charge we observed for Hsp90, Hsp70, and Hsp33 may be due to shared mechanisms of anti-aggregation activity.

Hsp90 was one of the most highly charged chaperones we analyzed. It is known to suppress the in vitro aggregation of numerous proteins including citrate synthase, amyloid beta, p53, and the nucleotide binding domain of the cystic fibrosis transmembrane conductance regulator (CFTR-NBD1).²²⁻²⁵ The ability of Hsp90 to suppress protein aggregation in vitro often does not require ATP.²⁵⁻²⁷ Hsp90 is an abundant protein under physiological conditions and its level increases in response to cell stress where the probability of protein mis-folding and aggregation is increased.^{28,29} Hsp90 can act as a holder chaperone, protecting mis-folded proteins from unfolding further and maintaining them in a folding-competent state.^{27,30}

The negative charge of Hsp90 is largely localized in two regions of the protein (Fig. 1a). The acid-rich charged linker (CL) is located between the structured N-terminal and middle domains of Hsp90. The other acidic region is located at the C-terminus and is referred to here as the C-terminal extension (CX). Both of these regions are believed to be unstructured based on amino acid composition and were deleted in the full-length crystal structure of Hsp90.³¹

Both highly charged regions of Hsp90 can be individually deleted without compromising essential chaperone function.³² Flexible regions at the ends of the acid-rich CL are required for essential chaperone function³³ and were retained in all constructs reported here. Hsp90, like many other chaperones,^{18,20} is believed to utilize hydrophobic surfaces to bind to exposed hydrophobic regions of mis-folded proteins.¹⁹ The acid-rich CL and CX (Fig. 1a) are among the least hydrophobic regions of Hsp90 (Fig. S1). Thus, deletion of the CL and CX is unlikely to interfere with Hsp90 binding to mis-folded proteins. These dispensable charge-rich regions provide a convenient system to investigate the role of charge in Hsp90 anti-aggregation activity.

The charge-rich regions of Hsp90 influence anti-aggregation activity

To test the role of charge in Hsp90 anti-aggregation activity, we generated constructs where the CL, the CX, or both were deleted. Hsp90 has previously been shown to reduce the aggregation of CFTR-NBD1, which is highly prone to aggregation at elevated temperatures (Fig. S2).^{25,34} Hsp90 constructs lacking either the charged linker (Δ CL) or the C-terminal extension (Δ CX) maintained near wild-type anti-aggregation activity (Fig. 2A), consistent with previous observations that deleting the charged linker from the N-domain resulted in only a modest impact on insulin aggregation.³⁵ However, the Hsp90 construct lacking both the charged linker and the C-terminal extension (Δ CL Δ CX) was inefficient at suppressing CFTR-NBD1 aggregation.

Deletion of the CL and CX reduces the net charge of Hsp90 and may therefore have unfavorable effects on the solubility of the chaperone. To test this possibility, we measured the propensity of our Hsp90 constructs to aggregate at elevated temperature in the absence of CFTR-NBD1. We found that deletion of the CL and the CX of Hsp90 does not impair the solubility of the chaperone under conditions where CFTR-NBD1 aggregates (Fig. 2a right panel, Fig. S3). These results indicate that the charge-rich regions of Hsp90 are important for suppressing CFTR-NBD1 aggregation, but not for the solubility of Hsp90.

We next investigated how charge-rich regions of Hsp90 affect the aggregation of a different mis-folded protein, citrate synthase (CS). Previous studies demonstrate that wild-type Hsp90 is able to suppress the temperature-induced aggregation of CS (Fig. S2).²² As observed for CFTR-NBD1, the Δ CL and Δ CX Hsp90 constructs maintained near wild-type anti-aggregation activity while Δ CL Δ CX was inefficient at suppressing CS aggregation (Fig. 2b). Under the conditions of the CS aggregation experiments, our Hsp90 constructs do not aggregate in the absence of CS (Fig. 2b right panel, Fig. S3). Together, these results indicate that the charge-rich regions are important for the anti-aggregation activity of Hsp90 for two different proteins.

Given the striking impact of deleting both charge-rich regions of Hsp90 on anti-aggregation activity, we wondered if Δ CL Δ CX was itself mis-folded. To address this question we analyzed the secondary structure by circular dichroism (CD). We find that Δ CL Δ CX contains secondary structure that is indistinguishable from wild-type Hsp90 and the individual Δ CL and Δ CX constructs (Fig. 3a). We next considered the possibility that deleting both charge-rich regions thermodynamically destabilized Hsp90. Thermodynamic stability can be measured by monitoring the concentration of urea required to unfold the

protein. We observed that similar concentrations of urea were required to unfold all of our constructs (Fig. 3b). These results indicate that the folded domains of $\Delta\text{CL}\Delta\text{CX}$ are well-folded and thermodynamically stable compared to wild-type Hsp90. It is also possible that deletion of the CL and CX disrupts the Hsp90 dimer, resulting in a non-functional monomeric construct. However, analytical ultracentrifugation indicates that $\Delta\text{CL}\Delta\text{CX}$ Hsp90 is dimeric (Fig. 3c).

Because our ΔCL constructs shorten the linker connecting the N-terminal and middle domains we wondered if interactions between these domains were perturbed. Communication between the N-terminal and middle domains is important for the inherent ATPase activity of Hsp90.³⁶ We tested ATPase activity and found that all of our constructs hydrolyze ATP at similar levels to wild-type Hsp90 (Fig. 3d). This result indicates that our deletions do not compromise communication between the N-terminal and middle domains. By deleting only the acid-rich region of the CL, our constructs retain flexibility that was previously found to be important for proper communication between the N-terminal and middle domains.^{33,37}

Our experimental observations are consistent with a model where both wild-type and $\Delta\text{CL}\Delta\text{CX}$ Hsp90 are capable of forming complexes with mis-folded proteins, but only wild-type Hsp90 complexes effectively suppress the aggregation of mis-folded proteins (Fig. 4a). It is well established that the propensity of proteins to aggregate increases under conditions that promote mis-folding.^{6,22–25} The ability of wild-type Hsp90 to suppress the temperature-induced aggregation of CFTR-NBD1 and CS indicates that it is capable of binding to these mis-folded proteins and reducing their propensity to aggregate. In our model, $\Delta\text{CL}\Delta\text{CX}$ Hsp90 is also capable of binding to mis-folded conformations, but is unable to suppress the aggregation of the mis-folded proteins. However, our results are also consistent with a model where $\Delta\text{CL}\Delta\text{CX}$ Hsp90 is defective for binding to mis-folded proteins.

We developed experiments to distinguish whether $\Delta\text{CL}\Delta\text{CX}$ is deficient for binding to mis-folded proteins or deficient in reducing the aggregation of bound, mis-folded proteins. It is challenging to measure the binding of Hsp90 to mis-folded proteins because of the inherent technical challenge of aggregation. However, the two possible models make different and testable predictions regarding the anti-aggregation activity of mixtures of wild-type and $\Delta\text{CL}\Delta\text{CX}$ Hsp90. If binding is the dominant defect of $\Delta\text{CL}\Delta\text{CX}$ Hsp90, then mixtures of wild-type and $\Delta\text{CL}\Delta\text{CX}$ Hsp90 should mimic the anti-aggregation activity of wild-type Hsp90 alone. If $\Delta\text{CL}\Delta\text{CX}$ competes with wild-type Hsp90 for binding to mis-folded protein, but does not protect mis-folded protein from aggregating, then mixtures of wild-type and $\Delta\text{CL}\Delta\text{CX}$ Hsp90 should exhibit intermediate anti-aggregation activity. Because Hsp90 is dimeric, there is the potential to form wild-type/ $\Delta\text{CL}\Delta\text{CX}$ Hsp90 heterodimers that would complicate the interpretation of these mixing experiments. To avoid this complication, we utilized a super-stabilized dimer (SSD) wild-type Hsp90 that does not heterodimerize with non-SSD Hsp90.³⁸ We find that mixtures of wild-type^{SSD} and $\Delta\text{CL}\Delta\text{CX}$ Hsp90 have anti-aggregation activity for both CFTR-NBD1 and CS that is intermediary to either Hsp90 construct alone (Fig. 4b and c). These results indicate that both wild-type and $\Delta\text{CL}\Delta\text{CX}$ Hsp90 are capable of binding to mis-folded proteins but only wild-type Hsp90 complexes effectively suppress the aggregation of mis-folded proteins.

Having established that $\Delta\text{CL}\Delta\text{CX}$ Hsp90 is capable of binding to mis-folded proteins, we next investigated if the $\Delta\text{CL}\Delta\text{CX}$ Hsp90 in these complexes aggregates. The efficiency of $\Delta\text{CL}\Delta\text{CX}$ Hsp90 aggregation from these complexes is likely to be influenced by the dynamics and mechanism of the aggregation process. Because $\Delta\text{CL}\Delta\text{CX}$ Hsp90 in isolation is soluble under the conditions of these experiments (Fig. 2), the relative amount of $\Delta\text{CL}\Delta\text{CX}$ Hsp90 aggregation should be controlled by kinetic competition between

aggregation and release from aggregating particles. To examine this issue, we monitored the content of aggregated particles formed by mis-folded CFTR-NBD1 in the presence and absence of $\Delta\text{CL}\Delta\text{CX}$ Hsp90 (Fig. 4d). Under the conditions of the experiment, we find that CFTR-NBD1 readily aggregates on its own and that this aggregation is efficiently rescued by wild-type Hsp90, but not $\Delta\text{CL}\Delta\text{CX}$ Hsp90 consistent with our light-scattering experiments (Fig. 3a). Aggregates formed in the presence of $\Delta\text{CL}\Delta\text{CX}$ Hsp90 show background levels of the chaperone, indicating that most $\Delta\text{CL}\Delta\text{CX}$ Hsp90 remains soluble. Thus, the mechanism and dynamics of the aggregation process permit $\Delta\text{CL}\Delta\text{CX}$ Hsp90 release.

The anti-aggregation activity of $\Delta\text{CL}\Delta\text{CX}$ can be rescued by an exogenous acid-rich region

If removal of charge caused the anti-aggregation defects of $\Delta\text{CL}\Delta\text{CX}$ Hsp90, adding charge back to the construct should rescue this activity. Alternatively, sequence-specific features of the CL and CX may have caused the loss of Hsp90 anti-aggregation activity. To distinguish these possibilities, we appended the human Hsp90 CL to the C-terminus of $\Delta\text{CL}\Delta\text{CX}$ to generate a charge rescue (CR) Hsp90 construct (Fig. 5a). While the charge-rich nature of the CL is evolutionarily conserved, it is one of the most diverse regions of Hsp90 with respect to sequence. In addition, by appending the human CL to the C-terminus, we have dramatically altered its physical location relative to the rest of the Hsp90 domains. We found that CR Hsp90 efficiently reduces CFTR-NBD1 (Fig. 5b) and CS (Fig. 5c) aggregation indicating that charge modulates the anti-aggregation activity of Hsp90.

Discussion

Our results clearly indicate that charge is important for the anti-aggregation activity of Hsp90. Hsp90 has been shown to reduce the aggregation propensity of numerous proteins.^{22–25} The aggregation propensity of many proteins, including CFTR and CS, increases under conditions that promote mis-folding. These observations are consistent with a widely recognized sequential model where natively-folded proteins transition to mis-folded states prior to aggregation (Fig. 4a). In this model, there are three potential protein conformations (native, mis-folded, and aggregated) that may bind to Hsp90.

Hsp90 binding to proteins in a mis-folded conformation is consistent with our observation that charge-rich regions of Hsp90 modulate the aggregation process. In our experiments, CFTR-NBD1 and CS aggregation was initiated by temperature stress, a condition that promotes mis-folding.³⁴ Mis-folded CFTR-NBD1 and CS are highly prone to aggregation, but this aggregation can be minimized by binding to Hsp90 (Fig. 2). The anti-aggregation activity of Hsp90 is impaired by deletion of the charge-rich regions indicating that charge plays an important role in Hsp90 anti-aggregation activity. Importantly, deletion of the charge-rich regions does not prevent Hsp90 from binding to mis-folded proteins (Fig. 4).

In bacterial Hsp90 (HtpG), the CL and CX regions are absent.^{35,39} However, in *Escherichia coli* HtpG the net charge remains highly negative due to the amino acid composition of the structured regions. The estimated charge per 100 amino acids on HtpG is -4.2 (compared to -2.2 for $\Delta\text{CL}\Delta\text{CX}$). These findings suggest that net charge may also be valuable in bacterial Hsp90 for anti-aggregation activity though the location of the charge is distinct from eukaryotic Hsp90.

There are two well-established physical properties of charge-rich regions that are likely to reduce aggregation: favorable interactions between charged atoms and aqueous solvent, and Coulombic repulsion between like-charged complexes. Our results indicate that the charge-rich regions of Hsp90 enhance the solubility of Hsp90 complexes with mis-folded proteins

through favorable solvent interactions and Coulombic self-repulsion with other like-charged complexes.

Net charge is a common characteristic of many chaperones and may influence their ability to prevent protein aggregation. We observe that net charge is a common characteristic of the Hsp33, Hsp70, and Hsp90 families of anti-aggregation chaperones. These chaperone families are also characterized by small hydrophobic patches capable of binding to exposed hydrophobic regions of mis-folded proteins. Our results with Hsp90 suggest that charge may be important in the function of these chaperones.

Materials and Methods

Sequence-based analysis of charge conservation

For the eukaryotic proteins Hsp90, Hsp70, Hsp60, and triosephosphate isomerase, protein sequences from the same six species were analyzed (Table S1). Predicted net charge of the proteins at pH 7 was determined based on the amino acid composition using the program SEDNTERP (Amgen). For the bacterial heat shock protein Hsp33, protein sequences from six species representing three distinct phyla were analyzed (Table S1). To ensure that the charge analysis was not affected by the diversity of bacterial genomes, only sequences that maintained at least 20 % sequence identity to the remaining sequences were analyzed.

Protein production

Hsp90 constructs were generated from the yeast *HSP82* gene with a 6×H tag (GGHHHHHHGGH) appended to the N-terminus and were expressed and purified essentially as described previously.⁴⁰ Following metal-affinity chromatography, samples were dialyzed into Buffer A (20 mM potassium phosphate pH 7.0, 1 mM ethylenedinitrilotetraacetic acid). Protein samples were purified further with anion exchange chromatography using a Source 15Q column (GE) eluted with a linear gradient from 0–1 M potassium chloride in Buffer A. Purified human CFTR-NBD1 protein was generously provided by Philip Thomas (University of Texas Southwestern Medical Center). Purified citrate synthase from porcine heart was purchased from Sigma-Aldrich.

CFTR-NBD1 Aggregation

Light scattering experiments were performed using a PTI QM-4SE spectrofluorometer (Photon Technology International) with a Neslab RTE-111 refrigerated circulating bath in a 0.3 cm pathlength cuvette. Aggregation of 1 μ M CFTR-NBD1 was measured in the presence of 5 μ M Hsp90 as described previously with native protein as the starting material.^{25,38} Protein samples were degassed for twenty minutes at room temperature and then right angle light scattering at a wavelength of 400nm was measured at 45 °C in Buffer B (100 mM Tris-hydrochloride pH 7.4, 0.385 M L-arginine, 10 mM dithiothreitol, 200 mM potassium chloride, 20 mM magnesium chloride). CFTR-NBD1 aggregation experiments were also attempted at varying concentrations of salt. However, CFTR-NBD1 aggregation in low salt buffers was too rapid to interpret data obtained under these conditions. All data were normalized to the light-scattering intensity for CFTR-NBD1 aggregation after twenty minutes at 45 °C.

The solubility of the Hsp90 constructs was monitored under the same conditions, in the absence of CFTR-NBD1. The solubility of wild-type and Δ CLACX Hsp90 was also analyzed by Native PAGE. Hsp90 protein samples (5 μ M) were heated to the indicated temperature for 5 minutes then cooled to room temperature. Samples were run on a 6% Native PAGE gel and stained with Sypro Ruby protein gel stain (Invitrogen).

Mixing experiments were performed by mixing 2.5 μM wild-type^{SSD} Hsp90³⁸ and 2.5 μM $\Delta\text{CL}\Delta\text{CX}$ Hsp90. Light scattering from the mixed sample was compared to that of CFTR-NBD1 in the presence or absence of 2.5 μM wild-type^{SSD} Hsp90 or 2.5 μM $\Delta\text{CL}\Delta\text{CX}$ Hsp90.

Citrate Synthase Aggregation

Aggregation of 0.075 μM CS was measured in the presence of 0.6 μM Hsp90 as described previously.²² Protein samples were degassed for forty minutes at room temperature and then right angle light scattering at a wavelength of 500nm was measured at 43 °C in Buffer C (40 mM HEPES pH 7.5, 1 mM magnesium chloride). The solubility of the Hsp90 constructs was monitored under the same conditions, in the absence of CS. Mixing experiments were performed by mixing 0.3 μM wild-type^{SSD} Hsp90 and 0.3 μM $\Delta\text{CL}\Delta\text{CX}$ Hsp90. Light scattering from the mixed sample was compared to that of CS in the presence or absence of 0.3 μM wild-type^{SSD} Hsp90 or 0.3 μM $\Delta\text{CL}\Delta\text{CX}$ Hsp90. All data were normalized to the light-scattering intensity for CS aggregation after forty minutes at 43 °C.

Circular Dichroism

CD measurements were made on a Jasco A-810 spectropolarimeter equipped with a Peltier temperature control unit in a 1 mm pathlength cuvette. To verify that the Hsp90 constructs were properly folded, the CD signal for 5 μM protein samples was monitored from 280 to 200 nm in Buffer A.

Urea Denaturation

Urea denaturation experiments were performed with 3 μM protein samples in a 0.3 cm pathlength cuvette. Samples were manually mixed, equilibrated for one hour, and changes in tryptophan fluorescence measured using a PTI QM-4SE spectrofluorometer with excitation set to 290 nm and emission scanned from 300 to 380 nm at 1 nm/second. Fraction folded was determined from the center-of-mass fluorescence after correction for the fluorescence of the folded and unfolded states as described previously.^{40,41} The data were then fit to a two state unfolding model using the program Kaleidograph.

Analytical Ultracentrifugation

Equilibrium analytical ultracentrifugation was performed essentially as described previously⁴⁰ except that the buffer consisted of 20 mM potassium phosphate pH 6.8, 150 mM potassium chloride, 5 mM magnesium chloride (Buffer D). Equilibrium experiments were performed at 20 °C on a Beckman XLI instrument using absorbance optics and a Ti60 rotor. 280nm absorbance scans were recorded at 12 hour intervals, and overlapping profiles were used as a criterion for equilibration. Experiments were performed with a protein concentration of 12 μM in Buffer D, a rotor speed of 8,000 RPM and an equilibration time of 36 hours.

Enzymatically Coupled ATPase Assay

ATP hydrolysis was enzymatically linked to NADH oxidation and monitored spectroscopically as described previously.^{40,42} ATPase assays were done at 37 °C with 3 μM Hsp90 and the ATPase components pre-warmed then mixed in a 1 cm pathlength cuvette. Measurements were taken at fifteen second intervals for ten minutes. Rates were determined by fitting the change in absorbance versus time to a linear model. Absorbance units were then converted to the molar amount of NADH oxidized and normalized to the concentration of Hsp90.

Analysis of protein aggregates

To monitor the content of the aggregates formed by mis-folded CFTR-NBD1, 200 μ L samples of CFTR-NBD1 in the presence and absence of Hsp90 were heated to 45 °C for 20 minutes in a water bath. Polycarbonate centrifuge tubes (Beckman) were pre-blocked with 10 mg/mL gelatin to prevent non-specific sticking then samples were pelleted at 17,000 rpm in a Beckman Optima TL Ultracentrifuge for 10 minutes. Pellets were washed with Buffer B and resuspended in SDS Loading Buffer. Samples were separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by western blot for Hsp90 or CFTR-NBD1 (graciously provided by William Balch, Scripps Research Institute – CFTR Folding Consortium). To compare protein levels on the Hsp90 and CFTR-NBD1 western blots, 10% or 100 % load samples were added.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful for the helpful suggestions of W. Kobertz and E. Mandon, to P. Thomas (University of Texas Southwestern Medical Center – CFTR Folding Consortium) for generously supplying purified CFTR-NBD1 protein, to William Balch for providing the CFTR-NBD1 antibody (Scripps Research Institute – CFTR Folding Consortium), and to C.R. Matthews for CD time. Analytical ultracentrifugation was performed at the University of Massachusetts Medical School Ultracentrifugation Facility with the aid of K. Crowley. This work was supported by grants to D.N.B. from the American Cancer Society (RSG-08-17301-GMC) and the National Institute of Health (R01-GM083038-01A).

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Fig. 1. Hsp90 constructs to test the role of charge-rich regions in anti-aggregation activity
(a) Schematic representation of *Saccharomyces cerevisiae* Hsp90. Hsp90 is composed of an N-terminal domain (N), charged-linker (CL), middle domain (M), C-terminal domain (C), and C-terminal extension (CX). The charge of Hsp90 is highly concentrated in the acid-rich CL and CX regions. (b) To test the role of these charge-rich regions in Hsp90 anti-aggregation activity, we made Hsp90 deletion constructs with reduced net charge.

Fig. 2. Charge-rich regions influence the anti-aggregation activity of Hsp90

(a) The combined deletion of both the CL and CX impaired the ability of Hsp90 to suppress CFTR-NBD1 aggregation at elevated temperature measured by right angle light scattering (left panel). In the absence of CFTR-NBD1, all Hsp90 constructs maintained wild-type solubility at 45 °C (right panel). (b) The combined deletion of both the CL and CX also impaired the ability of Hsp90 to suppress CS aggregation at elevated temperature measured by right angle light scattering (left panel). In the absence of CS, all Hsp90 constructs maintained wild-type solubility at 43 °C (right panel).

Fig. 3. Deletion of charge-rich regions does not disrupt the Hsp90 dimer or essential function

(a) CD analysis indicated that all Hsp90 constructs have secondary structure content similar to wild-type. (b) All Hsp90 constructs unfold at similar concentrations of urea. The midpoint concentration (C_m) of urea required to denature Hsp90 was unchanged by deletion of the CL and CX (Table S2). (c) Δ CL Δ CX Hsp90 behaves as a dimer during analytical ultracentrifugation. Single-species fit indicates that the molecular mass is 155 kDa (similar to the theoretical mass of a dimer: 151 kDa). The fit for a theoretical monomer is shown in grey for comparison. (d) Hsp90 constructs lacking the CL and CX were capable of hydrolyzing ATP similar to wild-type.

Fig. 4. Mechanistic model of charge-dependent anti-aggregation activity of Hsp90

(a) Stress-induced mis-folding of an aggregation-prone protein leads to exposure of hydrophobic surfaces that cause non-specific aggregation in the absence of a chaperone. In our model, both wild-type and $\Delta\text{CL}\Delta\text{CX}$ Hsp90 bind to the mis-folded state, but only wild-type complexes efficiently hinder aggregation. The deficient anti-aggregation activity of $\Delta\text{CL}\Delta\text{CX}$ Hsp90 could also be caused by impaired binding to mis-folded protein. (b and c) To determine whether $\Delta\text{CL}\Delta\text{CX}$ is unable to bind mis-folded proteins or is deficient for suppressing the aggregation of bound protein, we monitored the ability of an equimolar mixed sample of $\Delta\text{CL}\Delta\text{CX}$ and wild-type^{SSD} Hsp90 to suppress CFTR-NBD1 (b) or CS (c) aggregation. The intermediate anti-aggregation activity observed for the mixed sample indicates that both wild-type and $\Delta\text{CL}\Delta\text{CX}$ Hsp90 compete for binding to mis-folded proteins. (d) Analysis of CFTR-NBD1 aggregates formed in the presence and absence of Hsp90 variants. Aggregates were collected by centrifugation and analyzed by western blotting for Hsp90 and CFTR-NBD1. We find that most $\Delta\text{CL}\Delta\text{CX}$ Hsp90 remains soluble during the CFTR-NBD1 aggregation reaction.

Fig. 5. The anti-aggregation activity of $\Delta\text{CL}\Delta\text{CX}$ Hsp90 was rescued by an exogenous acid-rich region

(a) The human Hsp90 CL was appended to the C-terminus of $\Delta\text{CL}\Delta\text{CX}$ Hsp90 (black square) to generate a charge-rescue construct, CR. CR Hsp90 suppressed CFTR-NBD1 (b) and CS (c) aggregation at elevated temperature.

Table 1

Charge conservation of select cytoplasmic proteins

Molecular Chaperone	Average charge per 100 amino acids ^a
Hsp90	-5.0 ± 0.4
Hsp70	-2.5 ± 0.9
Hsp33	-5.6 ± 2.0
Hsp60	-1.1 ± 0.4
Triosephosphate Isomerase ^b	-0.7 ± 0.5

^a Average charge per 100 amino acids ± standard deviation, n=6.^b Non-chaperone cytoplasmic protein analyzed for comparison.