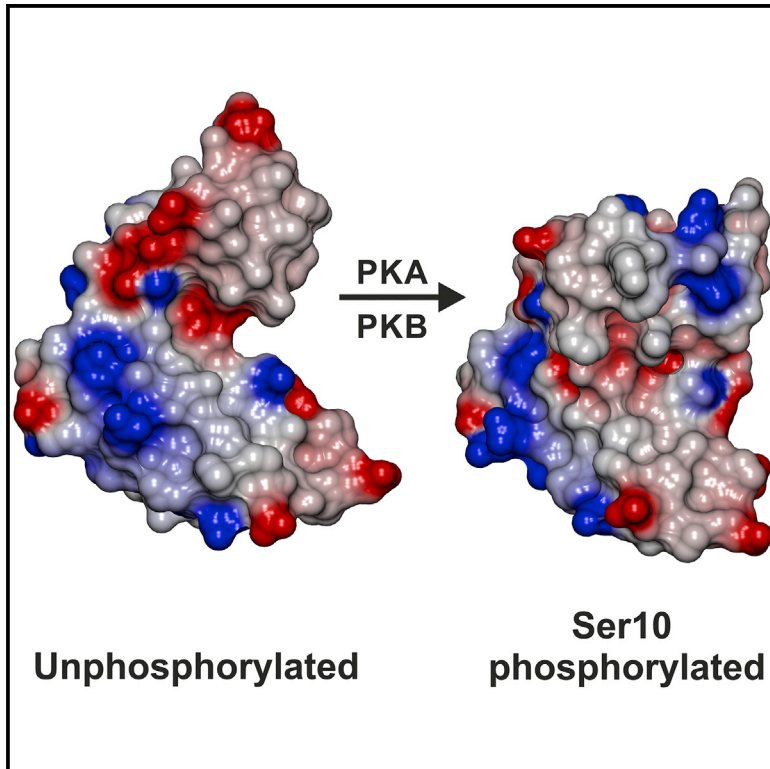


Structure

Phosphorylation of Cysteine String Protein Triggers a Major Conformational Switch

Graphical Abstract



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In Brief

Cysteine string protein (CSP) is phosphorylated in vivo on Ser10, and this modulates its protein interactions and effects on neurotransmitter release. Patel et al. report that Ser10 phosphorylation disrupts CSP's extreme N-terminal α helix, which triggers formation of a hairpin loop stabilized by ionic interactions between phosphoSer10 and the J-domain residue, Lys58.

Highlights

- First structure of a phosphorylated DnaJ/Hsp40 protein
- Phosphorylation destabilizes CSP's N-terminal α helix
- Newly disordered, phosphorylated N-terminal loop binds to the J domain
- Phosphorylation causes significant changes to CSP conformation and surface charge

Accession Numbers

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Phosphorylation of Cysteine String Protein Triggers a Major Conformational Switch

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SUMMARY

Cysteine string protein (CSP) is a member of the DnaJ/Hsp40 chaperone family that localizes to neuronal synaptic vesicles. Impaired CSP function leads to neurodegeneration in humans and model organisms as a result of misfolding of client proteins involved in neurotransmission. Mammalian CSP is phosphorylated *in vivo* on Ser10, and this modulates its protein interactions and effects on neurotransmitter release. However, there are no data on the structural consequences of CSP phosphorylation to explain these functional effects. We show that Ser10 phosphorylation causes an order-to-disorder transition that disrupts CSP's extreme N-terminal α helix. This triggers the concomitant formation of a hairpin loop stabilized by ionic interactions between phosphoSer10 and the highly conserved J-domain residue, Lys58. These phosphorylation-induced effects result in significant changes to CSP conformation and surface charge distribution. The phospho-switch revealed here provides structural insight into how Ser10 phosphorylation modulates CSP function and also has potential implications for other DnaJ phosphoproteins.

INTRODUCTION

CSP is a member of the DnaJ/Hsp40 family of molecular chaperone proteins. It is highly expressed in all neurons, where it localizes to synaptic vesicle membranes (Chamberlain and Burgoyne, 2000). Mammals express three CSP isoforms (α , β , γ), but CSP α is the major brain isoform and is the ortholog of the single CSP expressed in invertebrates. Human CSP α is encoded by the *DNAJC5* gene, mutations in which cause the neurodegenerative disorder, adult-onset dominant neuronal ceroid lipofuscinosis (Noskova et al., 2011). As mutations in CSP-encoding genes also cause neurodegeneration in flies (Zinsmaier et al., 1994), worms (Kashyap et al., 2014), and mice (Fernandez-Chacon et al., 2004), it is clear that CSP performs a universal neuroprotective function (Burgoyne and Morgan, 2015). CSP is widely thought to prevent neurodegeneration by promoting the correct conforma-

tion of presynaptic proteins involved in synaptic exo/endocytosis. Compelling evidence suggests that the SNARE protein SNAP-25 is one such protein, whose misfolding in the absence of CSP leads to neurodegeneration (Burgoyne and Morgan, 2011; Sharma et al., 2011, 2012). However, numerous other CSP-binding proteins have been suggested as functionally relevant client proteins for refolding, including the SNARE protein syntaxin (Chamberlain et al., 2001; Evans et al., 2001; Nie et al., 1999; Swayne et al., 2006); the calcium sensor synaptotagmin (Boal et al., 2011; Evans and Morgan, 2002); G protein subunits (Magga et al., 2000); and the endocytic protein dynamin (Zhang et al., 2012).

CSP has an evolutionarily conserved domain structure (Figure 1A). The J domain is a signature of the DnaJ/Hsp40 family of molecular chaperones, which bind misfolded proteins and recruit/activate the 70 kDa heat shock cognate protein (Hsc70/Hsp70) to regulate protein folding (Hennessy et al., 2005). Indeed, CSP binds Hsc70 and stimulates its ATPase activity, and prevents aggregation of denatured proteins (Braun et al., 1996; Chamberlain and Burgoyne, 1997a, 1997b). All other domains are unique to CSP homologs. The cysteine string domain comprises 13–15 cysteine residues in an approximately 25-amino-acid motif, most of which are palmitoylated (Gundersen et al., 1994). This domain is essential for targeting CSP to synaptic vesicles and for neurotransmitter release *in vivo* (Arnold et al., 2004; Chamberlain and Burgoyne, 1998; Greaves and Chamberlain, 2006; Ohyama et al., 2007; Stowers and Isacoff, 2007). The function of the linker region connecting the J domain to the cysteine string is unclear, as mutation of this domain has relatively mild effects on CSP phenotypes (Arnold et al., 2004; Bronk et al., 2005; Zhang et al., 1999), although it may regulate binding to synaptotagmin (Boal et al., 2011). The C-terminal domain displays relatively low sequence conservation among CSP homologs from various species; and its function is poorly understood. Finally, CSPs contain a short N-terminal polypeptide sequence that is phosphorylated *in vivo* from worms to humans (Collins et al., 2005; Evans and Morgan, 2005; Evans et al., 2001; Hilger et al., 2009; Zielinska et al., 2009). Phosphorylation of mammalian CSP α on Ser10 inhibits binding to syntaxin and synaptotagmin, but not Hsc70, (Evans and Morgan, 2002; Evans et al., 2001) and modulates cellular exocytosis release kinetics (Chiang et al., 2014; Evans et al., 2001). However, there are no data on how Ser10 phosphorylation affects CSP structure to bring about these functional changes. Here we report the nuclear magnetic resonance (NMR) structures of the CSP N terminus in both the unphosphorylated and phosphorylated states.

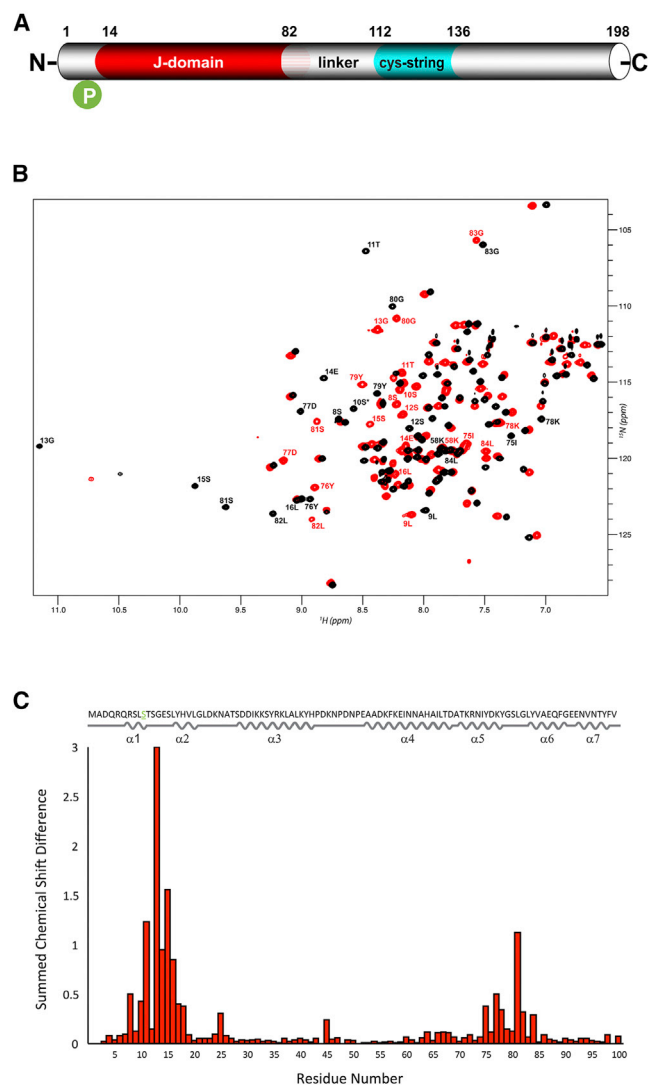


Figure 1. NMR Analysis of Unphosphorylated and Phosphorylated CSP₁₋₁₀₀

(A) Domain structure of CSP.

(B) ¹H-¹⁵N HSQC spectra of CSP₁₋₁₀₀ (red) and pCSP₁₋₁₀₀ (black). The HSQC spectra shows well-resolved, non-overlapping peaks indicating both CSP₁₋₁₀₀ and pCSP₁₋₁₀₀ are folded. Upon phosphorylation, chemical shift dispersion can be observed for the indicated residues around Ser10 and Ser81.

(C) Chemical shift differences ($\Delta\delta$) between CSP₁₋₁₀₀ and pCSP₁₋₁₀₀ amide resonances, calculated using $\Delta\delta = [(\delta_{\text{H}})^2 + (\delta_{\text{HN}} \cdot 0.15)^2]^{1/2}$. The amino acid sequence and secondary structure elements of CSP₁₋₁₀₀ obtained from the NMR structure are shown at the top of the figure.

RESULTS

Generation of Soluble, Monomeric CSP Constructs for NMR

To investigate the structural consequences of phosphorylation on mammalian CSP α , we purified bacterially expressed recombinant proteins for analysis. Full-length CSP₁₋₁₉₈ formed mixed oligomers of >239 kDa, based on analytical ultracentrifugation (AUC) analysis (Figure S1A), representing at least

Table 1. NMR and Refinement Statistics for Protein Structures

	CSP 1-100	pCSP 1-100
NMR Distance and Dihedral Constraints		
Distance constraints		
Total NOE	2,450	3,120
Intra-residue	851	968
Inter-residue	1,599	2,152
Sequential ($ i - j = 1$)	655	839
Medium-range ($2 \leq i - j \leq 4$)	530	758
Long-range ($ i - j \geq 5$)	414	555
Total dihedral angle restraints	187	181
ϕ	92	90
ψ	95	91
Structure Statistics		
Violations (mean and SD)		
Distance constraints (Å)	0.08 ± 0.06	0.07 ± 0.07
Dihedral angle constraints (°)	1.15 ± 0.88	1.44 ± 1.10
Max. dihedral angle violation (°)	4.79	5.57
Max. distance constraint violation (Å)	0.72	1.13
Deviations from idealized geometry		
Bond lengths (Å)	0.0041 ± 0.00013	0.0050 ± 0.0001
Bond angles (°)	0.59 ± 0.012	0.65 ± 0.016
Impropers (°)	1.54 ± 0.056	1.59 ± 0.067
Average pairwise root-mean-square deviation^a (Å)		
Heavy	0.47	0.49
Backbone	0.14	0.20
Ramachandran statistics^b		
Most favored/additionally allowed/generously allowed (%)	88.2/11.8/0.0	80.6/18.3/1.1

^aStatistics are calculated and averaged over an ensemble of the 20 lowest-energy water-refined structures out of 100 calculated structures.

^bRamachandran statistics calculated using PROCHECK.

ten subunits based on the predicted monomeric mass of 23.5 kDa. In contrast, the C-terminal domain construct CSP₁₃₇₋₁₉₈ was monodisperse with an estimated molecular mass of 9.0 kDa, close to its predicted monomeric mass of 8.2 kDa (Figure S1B). The heteronuclear single quantum coherence (HSQC) spectrum for ¹⁵N-labeled CSP₁₃₇₋₁₉₈ shows poor ¹H chemical shift dispersion, with most resonances appearing between 7.9 and 8.6 ppm, indicating that the C-terminal domain is essentially unstructured (Figure S1C). It has been suggested that CSP's tendency to aggregate may be due to the cysteine string domain (Swayne et al., 2003). However, mutation of all 14 cysteines to serines in full-length CSP₁₋₁₉₈ did not reduce oligomerization (Figure S1D), and a CSP₁₋₁₁₂ construct that lacks the entire cysteine string precipitated into visible aggregates. In contrast, CSP₁₋₁₀₀ was monomeric with well-dispersed resonances in the ¹H-¹⁵N HSQC spectra (Figure 1B). Further structural work was therefore performed using CSP₁₋₁₀₀.

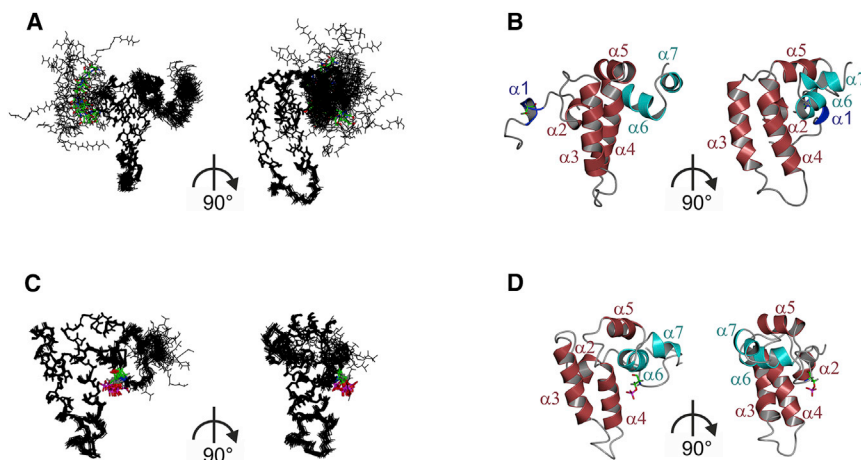


Figure 2. Structures of Unphosphorylated and Phosphorylated CSP₁₋₁₀₀

Ensembles (A and C) and ribbon representations (B and D) of the lowest-energy conformers of CSP₁₋₁₀₀ (A and B) and pCSP₁₋₁₀₀ (C and D); two different views differing by 90° are shown for each, with Ser10 represented as sticks. For the ensembles, all main-chain heavy atoms for 20 structures are displayed (see also Figure S2 for C α backbone ensembles). For the ribbon representations, helices are highlighted in purple (α 1), red (α 2– α 5), or cyan (α 6– α 7).

Solution Structure of CSP₁₋₁₀₀ in the Non-phosphorylated State

Using conventional triple-resonance NMR spectra, the backbone resonances for 99 of 100 residues of CSP₁₋₁₀₀ were assigned, and the structure was determined with 2,637 distance and dihedral angle restraints (Table 1). This revealed a secondary structure consisting of seven α helices, α 1(7–10), α 2(16–20), α 3(28–42), α 4(52–68), α 5(70–78), α 6(83–90), and α 7(93–98) (Figure 1C). These secondary structure elements are well defined, although helices α 1, α 6, and α 7 are much less converged than helices α 2– α 5 due to the lack of stabilizing helix-helix interactions in the tertiary structure (Figures 2A, 2B, and S2A). Helix α 1 is a short α helix located in an otherwise highly flexible, unstructured N-terminal region; helices α 2– α 5 comprise the autonomously folded J domain; and helices α 6 and α 7 are located in the linker region C-terminal to the J domain. The secondary structure and overall fold of helices α 2– α 5 strongly resemble other previously determined J-domain structures, such as yeast Sis1p (PDB: 4RWU; Figure S3A). Our CSP₁₋₁₀₀ structure is also similar to the structure deposited by the RIKEN Structural Genomics Consortium of a CSP₅₋₁₀₀ construct (PDB: 2CTW; Figure S3B), although clear differences are apparent in the non-J-domain helices: α 1, α 6, and α 7. This is especially evident in the N-terminal α 1 helix, which is not helical in any of the 20 submitted 2CTW structures. It is likely that the first four residues of CSP, which are absent in the 2CTW construct, are important for α 1 helix formation.

A Phosphorylation-Induced Conformational Switch

The α 1 helix contains the Ser10 residue, which is phosphorylated in vivo and which modulates CSP's cellular functions (Collins et al., 2005; Evans and Morgan, 2005; Evans et al., 2001). To gain insight into how phosphorylation affects CSP structure, purified ¹³C/¹⁵N CSP₁₋₁₀₀ was incubated with MgATP and protein kinase A (PKA). Parallel incubation using unlabeled proteins showed that under these conditions, rapid and efficient phosphorylation on only Ser10 was achieved, as determined by γ ³²-ATP incorporation and mass spectrometry (Figure S4). The incubation mixture containing ¹³C/¹⁵N CSP₁₋₁₀₀, MgATP, and PKA was used without further purification for structure determination. Triple-resonance heteronuclear NMR spectroscopy with non-uniform sampling (NUS)

was then performed, allowing full data collection for spectral assignment in a short space of time. The spectra revealed significant changes to the chemical shifts for various residues, notably those around Ser10 and Ser81 (Figures 1B and 1C). Based on the mass spectrometry data, the chemical shift effects around S10 are a direct result of Ser10 phosphorylation, whereas those around Ser81 indicate a possible structural change in the loop connecting helices α 5 and α 6. Backbone resonance assignments for all 100 assignable amino acid residues were obtained, and the structure of pCSP₁₋₁₀₀ was calculated using 3,301 distance and dihedral angle restraints. Strikingly, the structure of serine10-phosphorylated CSP₁₋₁₀₀ reveals an order-to-disorder transition in the conformation of helix α 1, which in turn triggers the interaction of the newly disordered N terminus with the J-domain helix α 4 (Figures 2C, 2D, and S2B). This conformational phospho-switch results in a more compact overall structure of pCSP₁₋₁₀₀ with significantly altered surface charge distribution (Figures 3A and 3B). Notably, the ionic interaction between the negatively charged phosphate group on phospho-Ser10 and the positively charged ϵ -amino group of Lys58 stabilizes and sequesters the N-terminal region of CSP (Figures 3C and 3D), which also brings the N-terminal region into much closer proximity to Ser81, hence, explaining the significant chemical shift changes in this region. The interaction between phospho-Ser10 and Lys58 is corroborated by the observation of a network of nuclear Overhauser effects (NOEs) involving the surrounding residues, including phospho-Ser10 to Ser81/Leu82, and Val19 to Glu59/Ile60/Ala63. Unambiguous direct NOEs between phospho-Ser10 and Lys58 are not observed, as the distances between the non-exchangeable protons in the two residues are over 5 Å and, hence, expected to give rise to very weak NOEs. The relatively small chemical shift change in the ¹⁵N-HSQC spectrum around residue Lys58 compared with Ser81 is explained by the lack of conformational change in helix α 4.

DISCUSSION

The conformational phospho-switch reported here provides a structural basis for the previously established effects of Ser10 phosphorylation on CSP function. By destabilizing the N-terminal α 1 helix and reducing its accessibility, phosphorylation would weaken protein-protein interactions involving this region, potentially explaining how Ser10 phosphorylation reduces CSP

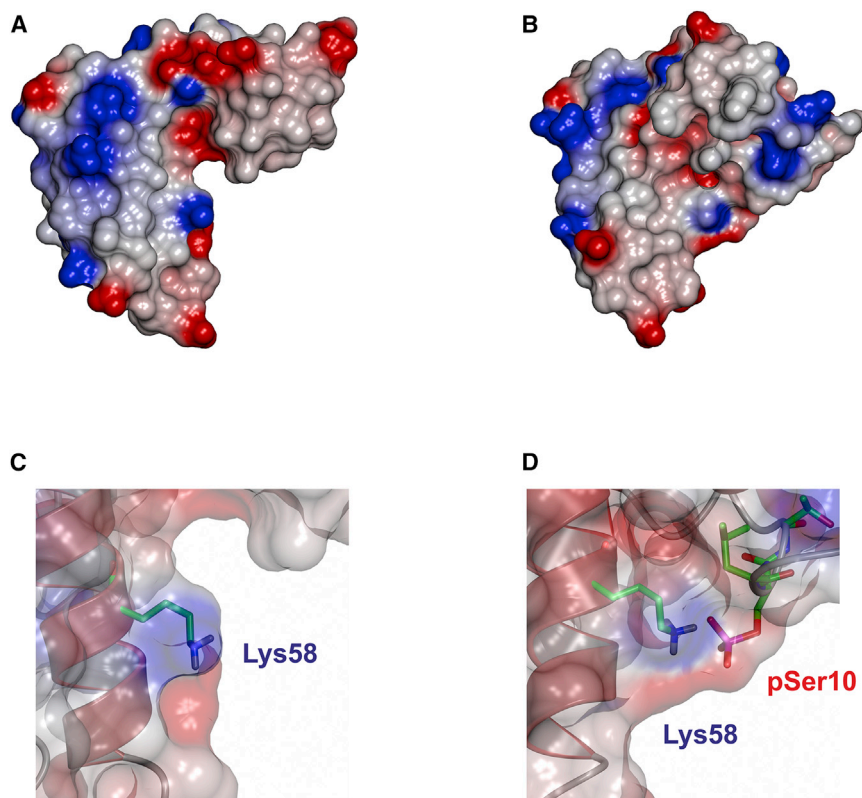


Figure 3. Phosphorylation of Ser10 Triggers a Conformational Switch

(A and B) Surface representation of the lowest-energy conformers of CSP₁₋₁₀₀ and pCSP₁₋₁₀₀. (C) Close-up view of Lys58, represented as sticks, showing the surface-exposed positively charged patch in CSP₁₋₁₀₀. (D) Phosphorylation triggers the interaction of phospho-Ser10 and Lys58, altering the surface charge distribution in this region.

binding to syntaxin and synaptotagmin (Evans and Morgan, 2002; Evans et al., 2001). In contrast, the structure of the J domain and the accessibility of the HPD motif required for Hsp70 activation are unaffected by Ser10 phosphorylation (Figure 4A), thus revealing why CSP phosphorylation has no effect on Hsp70 interactions (Evans et al., 2001). Finally, the new interface created jointly by the phosphorylated N terminus and α 4 helix (Figure 3B) provides a novel scaffold for protein and/or lipid interactions that could explain the effects of Ser10 phosphorylation on fusion pore expansion during exocytosis (Chiang et al., 2014; Evans and Morgan, 2002; Evans et al., 2001; Prescott et al., 2008).

Phosphorylation-induced order/disorder transitions, as shown here for CSP, are becoming increasingly recognized as regulatory switches that control protein function. For example, phosphorylation of retinoblastoma protein on Ser608 causes the disordered loop containing this residue to interact with the binding pocket for the E2F transactivation domain, thus inhibiting E2F binding (Burke et al., 2012). In addition, multi-site phosphorylation of folded pentameric nucleophosmin has been shown to cause electrostatic repulsion between the protomers and a transition to unfolded monomers, thereby destabilizing binding sites that exist in the oligomeric protein (Mitrea et al., 2014). Finally, a phosphorylation-induced disorder-to-order transition in 4E-BP2 has recently been shown to reduce eIF4E binding by sequestering a helical binding motif into a β strand (Bah et al., 2015).

The N-terminal domain of CSP is phosphorylated in vivo in humans, rodents, flies, and worms (Collins et al., 2005; Evans and Morgan, 2005; Evans et al., 2001; Hilger et al., 2009; Zielinska et al., 2009), indicating that phospho-regulation of CSP is as

evolutionarily conserved as its role in preventing neurodegeneration. Given that 36 of the 41 DnaJ proteins encoded by the human genome are serine/threonine phosphorylated (Hornbeck et al., 2015), the CSP phospho-switch revealed here could be a general mechanism for conformational regulation of DnaJ/Hsp40 chaperones. Indeed, the Lys58 residue that interacts with phospho-Ser10 in CSP has long been recognized to be among the most highly conserved residues in DnaJ proteins (Hennessy et al., 2005) (Figure 4B), although the reason for this conservation has been unclear. Furthermore, Lys58 in CSP is a ubiquitination site (Wagner et al., 2011), as are the orthologous

Lys residues in human DNAJA1 and DNAJB1. The close interaction of phospho-Ser10 with Lys58 revealed here would likely impede access by E3 ligases, thereby antagonizing CSP ubiquitination. Given that phosphorylation of 4E-BP2 has recently been shown to inhibit Lys57 ubiquitination by triggering a disorder-to-order transition (Bah et al., 2015), the phospho-switch reported here may represent an alternative mechanism for regulating protein conformation by reciprocally antagonistic posttranslational modifications.

EXPERIMENTAL PROCEDURES

Expression and Purification of CSP

Full-length CSP₁₋₁₉₈ in the pQE30 vector (QIAGEN) has been previously described (Evans et al., 2001) and was used to prepare the CSP14CS, CSP₁₃₇₋₁₉₈, and CSP₁₋₁₁₂ constructs via site-directed mutagenesis. CSP₁₋₁₀₀ was synthesized (Geneart; Life Technologies) based on the human coding sequence and codon optimized for expression in *Escherichia coli* and subcloned into the pE-SumoPro Kan expression vector (LifeSensors). Expression of recombinant CSP was induced in *E. coli* BL21 Star (Invitrogen) competent cells using 1 mM isopropyl β -D-1-thiogalactopyranoside at 18°C for 18 hr. Uniformly isotope-labeled CSP was expressed in M9 minimal media with ¹⁵NH₄Cl and/or ¹³C₆-glucose as the sole nitrogen and carbon sources, respectively. Cells were harvested by centrifugation and resuspended in lysis buffer containing 20 mM Tris (pH 7.5), 500 mM NaCl, 20 mM imidazole with protease inhibitors (complete mini EDTA-free protease inhibitor cocktail tablets; Roche). After lysis by cell disruption, the soluble fraction was isolated by centrifugation at 27,000 \times g for 45 min. The supernatant was applied to a charged HisTrap FF 5 ml affinity column (GE Healthcare), washed with 20 mM Tris (pH 7.5), 500 mM NaCl, 50 mM imidazole, and purified protein eluted with a linear imidazole gradient from 50 mM to 500 mM. The His-SUMO tag on CSP₁₋₁₀₀ was removed by incubation with recombinant ULP-1 overnight at 4°C. The CSP₁₋₁₀₀ protein was subjected to gel filtration through a Superdex-75 column (GE Healthcare)

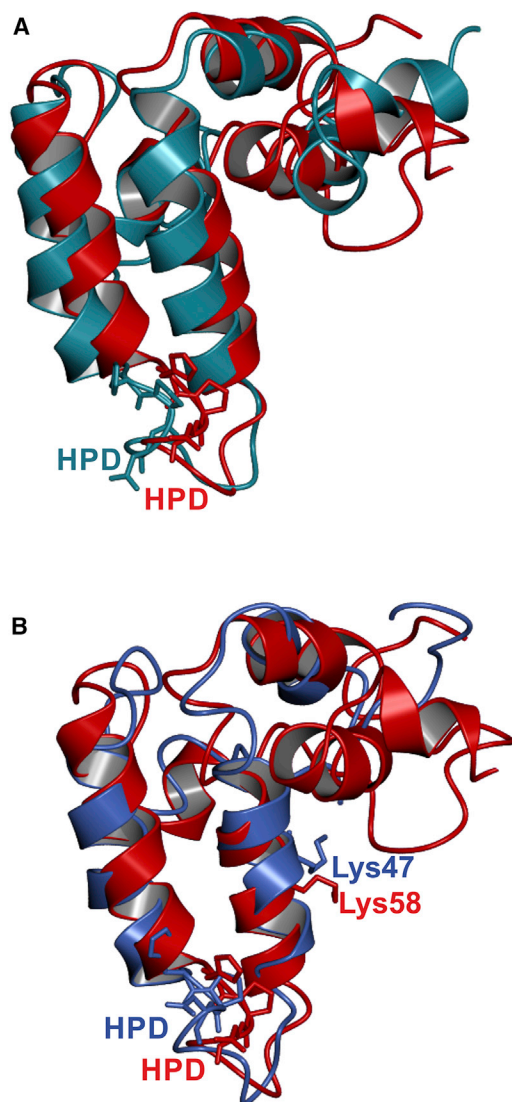


Figure 4. Implications of CSP Phosphorylation for Interactions with Hsc70 and Ubiquitin Ligases

(A) Overlay of CSP₁₋₁₀₀ (blue) and pCSP₁₋₁₀₀ (red), with the conserved HPD motif represented as sticks.

(B) Overlay of *E. coli* DnaJ (PDB: 1BQ0; blue) with human pCSP₁₋₁₀₀ (red), with the conserved Lys58 and Lys48 residues, respectively, and HPD motifs represented as sticks.

equilibrated with 20 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.5), 150 mM NaCl.

In Vitro Phosphorylation

Purified CSP₁₋₁₀₀ was phosphorylated by mixing in a 340:1 molar ratio with protein kinase A catalytic subunit (Sigma-Aldrich), 1 mM DTT, 10 mM MgCl₂, 0.5 mM EDTA, and 1 mM ATP. For analysis of phosphorylation kinetics, mixtures were supplemented with 3 μ Ci of radiolabeled γ -³²P-ATP per 50 μ l reaction and incubated for various times before stopping the reaction by addition of boiling 2 \times Laemmli buffer (Sigma-Aldrich). Samples were run on pre-cast Novex SDS-PAGE gels (Invitrogen), stained with Coomassie blue, dried, and exposed to phosphor screens overnight before imaging on a Phosphorimager Si (GE Healthcare). For NMR spectroscopy and mass spectrometry analyses, in vitro phosphorylation mixtures were prepared using non-radiolabeled ATP and incubated for 4 hr to ensure the reaction was complete.

Estimation of Native Molecular Mass

Analytical ultracentrifugation was performed at the Astbury Center for Structural Molecular Biology, University of Leeds. CSP protein samples were spun at 50,000 rpm at 20.1°C for 9 hr for sedimentation velocity analysis, during which 98 absorbance scans at 279 nm were performed and used to estimate the native molecular mass. Size-exclusion chromatography-multiple-angle laser light scattering analysis was performed using a Dawn Helelos instrument at a laser wavelength of 658 nm.

Mass Spectrometry

Phosphorylation site mapping was performed at the FingerPrints' Proteomics Facility, University of Dundee. PKA-phosphorylated CSP₁₋₁₀₀ protein was separated by SDS-PAGE, digested with trypsin, and then extracted before being applied to an nLC liquid chromatography system (Dionex/LC Packings) coupled to a 4000 QTRAP mass spectrometer (Applied Biosystems/Sciex). Mass spectrometry data were filtered by removing missed cleavages and employing a 1% false discovery rate.

NMR Spectroscopy

All spectra were acquired at 298 K on Bruker Avance III 600 MHz and 800 MHz spectrometers. For non-phosphorylated CSP₁₋₁₀₀, sequence-specific backbone resonance assignment was obtained using standard multidimensional heteronuclear NMR experiments: HNCA, HN(CO)CA, HNCACB, CBCA(CO)NH, HNCO, HNCACO, HBHANH, HBHA(CO)NH. Side-chain assignments were obtained from a 3D HCCH total correlation spectroscopy (HCCH-TOCSY) experiment. NOEs were derived from 3D ¹⁵N- and ¹³C-edited NOE spectroscopy (NOESY)-HSQC experiments with 130 ms mixing time. For pCSP₁₋₁₀₀, sequence-specific backbone resonance assignment was obtained using the multidimensional heteronuclear NMR experiments as described above, with NUS. Side-chain assignments were obtained from a 3D HCCH-TOCSY experiment. NOEs were derived from 3D ¹⁵N- and ¹³C-edited NOESY-HSQC experiments with 140 ms mixing time.

NMR Assignments and Structure Calculations

All NMR spectra were processed with TopSpin (Bruker) and analyzed using the CCPN Analysis package (Vranken et al., 2005). Backbone torsion angles were derived from analysis of H α , C α , C β , and C' chemical shifts using the DANGLE program (Cheung et al., 2010). All structure calculations were carried out using the Aria package (Rieping et al., 2007) with the IUPAC PARALLHDGv5.3 and TOPALLHDGv5.3 parameter sets. Structural statistics are summarized in Table 1.

ACCESSION NUMBERS

Coordinates and chemical shifts have been deposited in the PDB and Biological Magnetic Resonance Bank under accession codes PDB: 2N05 and BMRB: 25515 for CSP₁₋₁₀₀, and PDB: 2N04 and BMRB: 25514 for pCSP₁₋₁₀₀.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.str.2016.06.009>.

AUTHOR CONTRIBUTIONS

P.P., G.R.P., and A.M. performed protein purification and biochemical experiments; P.P. and L.Y.L. performed NMR experiments; P.P. performed structure calculations; P.P., L.Y.L., R.D.B., and A.M. analyzed and interpreted the data. A.M., L.Y.L., and R.D.B. conceived and designed the study. P.P. and A.M. wrote the manuscript with input from all authors.

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Structure, Volume 24

Supplemental Information

Phosphorylation of Cysteine String Protein

Triggers a Major Conformational Switch

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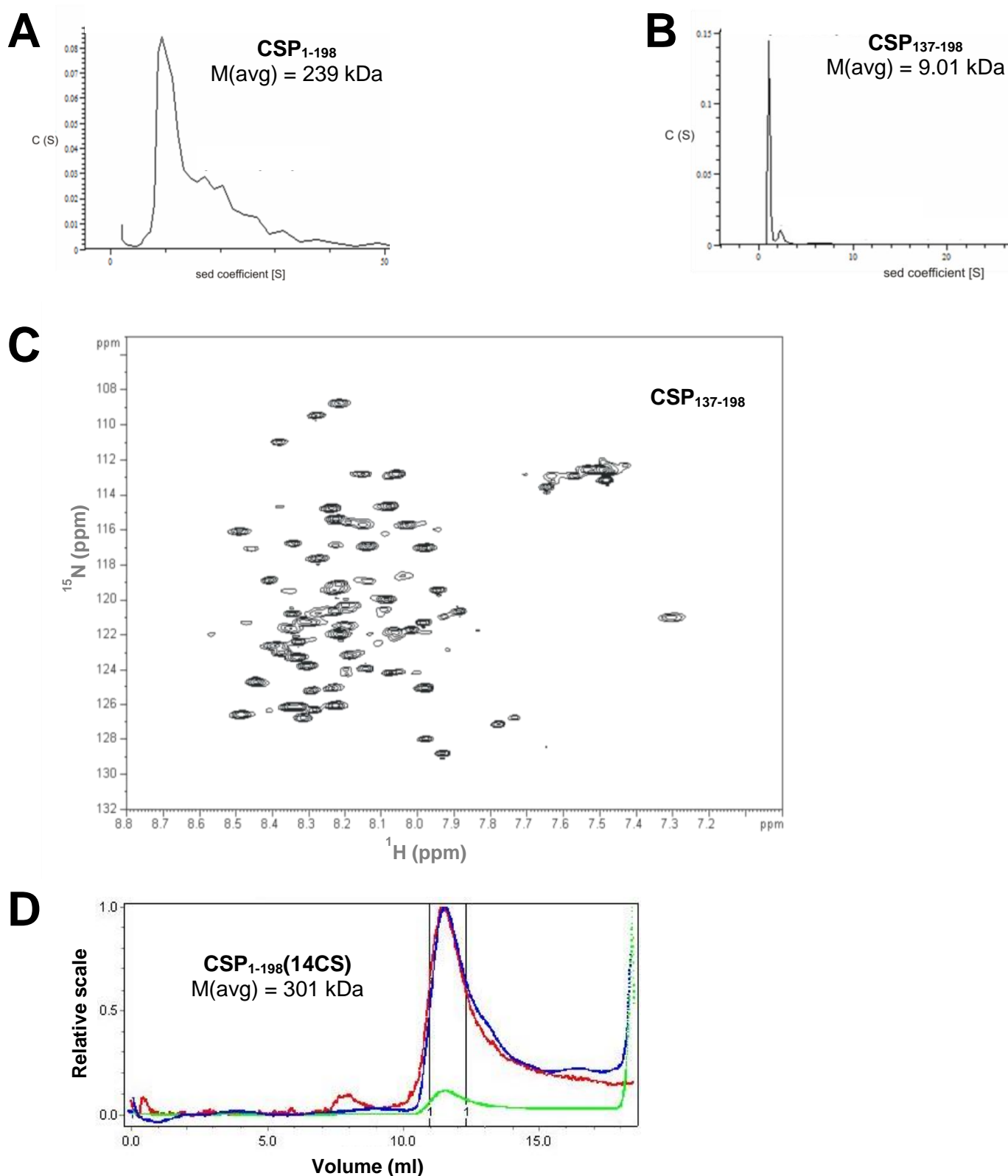


Fig. S1, related to Fig. 1: Characterisation of recombinant CSP constructs

a) Analytical ultracentrifugation of full length CSP₁₋₁₉₈. b) Analytical ultracentrifugation of CSP₁₃₇₋₁₉₈. c) ¹H-¹⁵N HSQC spectra of CSP₁₃₇₋₁₉₈. d) Size exclusion chromatography - multiple angle laser light scattering analysis of full length CSP₁₋₁₉₈ with all 14 cysteines mutated to serines (14CS).

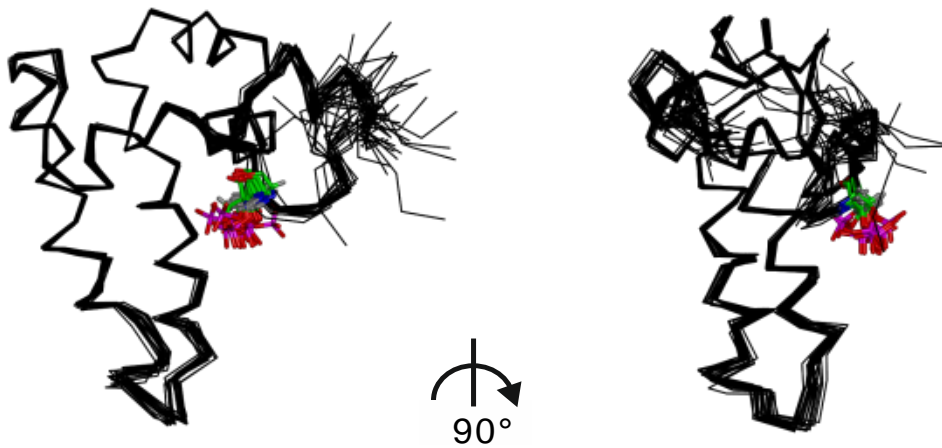
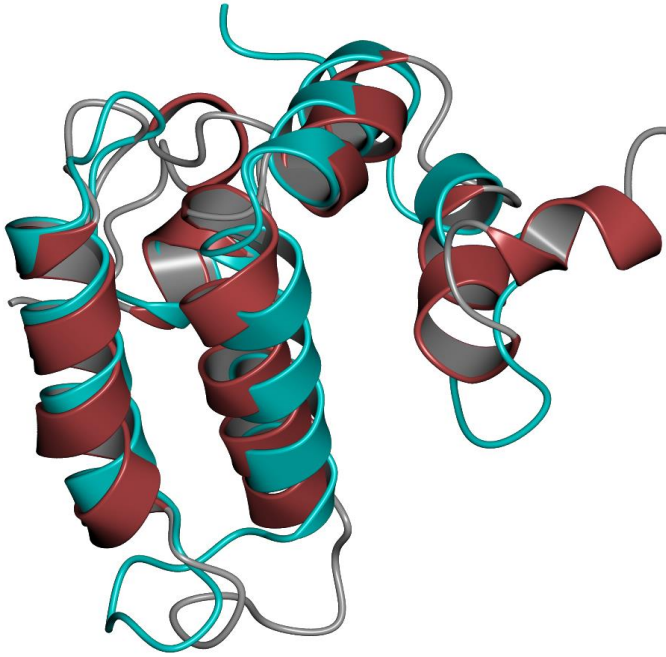
A**B**

Fig. S2, related to Fig. 2: Structures of unphosphorylated and Ser10-phosphorylated CSP₁₋₁₀₀.

Ensembles of the 20 lowest energy conformers of CSP₁₋₁₀₀ (a) and pCSP₁₋₁₀₀ (b) displayed as C α backbone representations; two different views differing by 90° are shown for each, with Ser10 represented as sticks.

A



B

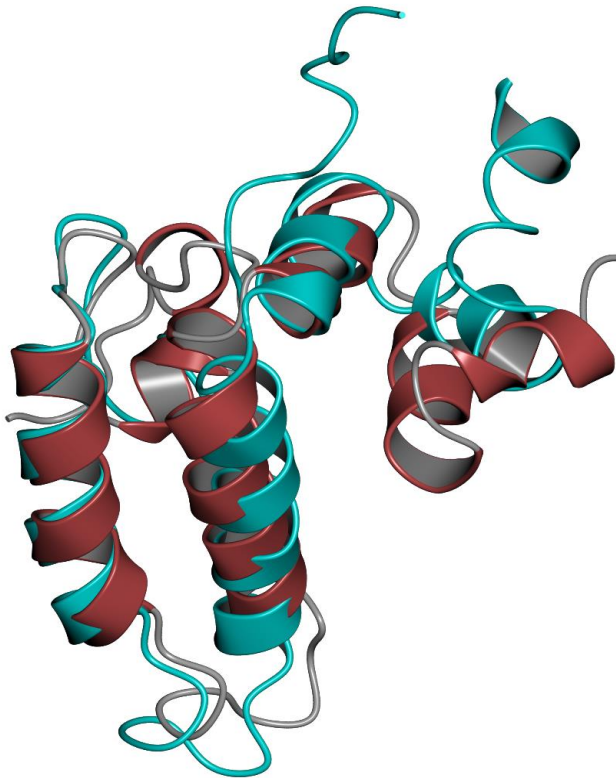
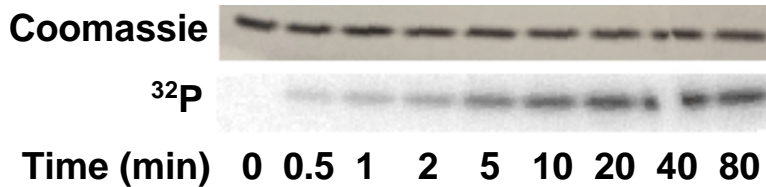


Fig. S3, related to Fig. 2: Structural similarities between CSP₁₋₁₀₀ and DnaJ/Hsp40 proteins in the unphosphorylated state.

a) Overlay of the lowest energy conformer of CSP₁₋₁₀₀ (red) with the J domain of yeast Sis1 protein (PDB code: 4RWU, cyan). b) Overlay of the lowest energy conformer of CSP₁₋₁₀₀ (red) with CSP₅₋₁₀₀ (PDB code: 2CTW, blue).

A**B**

Sequence	# PSMs	Modifications	IonScore	Exp Value	Charge	MH+ [Da]	ΔM [ppm]	RT [min]	# Missed Cleavages
SLsTSGESLYHVLGLDK	153	S10(Phospho)	63	0.000	2	1885.895	0.31	67.11	0
sLsTSGESLYHVLGLDK	33	N-Term(Acetyl); S10(Phospho)	50	0.000	2	1927.906	0.52	77.20	0
sLsTSGESLYHVLGLDK	33	S8(Phospho); S10(Phospho)	40	0.002	2	1965.861	0.16	76.41	0

Fig. S4, related to Fig. 3: *in vitro* phosphorylation of CSP₁₋₁₀₀.

a) Kinetics of γ^{32} -ATP incorporation following incubation of CSP₁₋₁₀₀ with protein kinase A and radiolabelled MgATP. Samples were run on SDS-PAGE and visualised by Coomassie Blue staining (*Coomassie*, upper panel) and phosphorimaging (32 P, lower panel). Images shown are cropped to remove unnecessary blank space. b) Mass spectrometric identification of phosphorylation sites on CSP₁₋₁₀₀ after incubation with protein kinase A and MgATP.