Structure and Functional Studies of the CS Domain of the Essential H/ACA Ribonucleoparticle Assembly Protein SHQ1*

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Abstract

H/ACA ribonucleoprotein particles are essential for ribosomal RNA and telomerase RNA processing and metabolism. Shq1p has been identified as an essential eukaryotic H/ACA small nucleolar (sno) ribonucleoparticle (snoRNP) biogenesis and assembly factor. Shq1p is postulated to be involved in the early biogenesis steps of H/ACA snoRNP complexes, and Shq1p depletion leads to a specific decrease in H/ACA small nucleolar RNA levels and to defects in ribosomal RNA processing. Shq1p contains two predicted domains as follows: an N-terminal CS domain and a C-terminal region of high sequence homology called the Shq1 domain. Here we report the crystal structure and functional studies of the Saccharomyces cerevisiae Shq1p CS domain. The structure consists of a compact anti-parallel β-sandwich fold that is composed of two β-sheets containing four and three β-strands, respectively, and a short α-helix. Deletion studies showed that the CS domain is required for the essential functions of Shq1p. Point mutations in residues Phe-6, Gln-10, and Lys-80 destabilize Shq1p in vivo and induce a temperature-sensitive phenotype with depletion of H/ACA small nucleolar RNAs and defects in rRNA processing. Although CS domains are frequently found in co-chaperones of the Hsp90 molecular chaperone, no interaction was detected between the Shq1p CS domain and yeast Hsp90. These results show that the CS domain is essential for Shq1p function in H/ACA snoRNP biogenesis in vivo, possibly in an Hsp90-independent manner.

Modification of uridine to pseudouridine in ribosomal RNA and some spliceosomal RNAs is catalyzed by highly specialized ribonucleoparticle (RNP) complexes called box H/ACA RNPs (1−5). Depending on their site of maturation and action H/ACA RNPs are classified into two classes, small nucleolar RNPs (snoRNPs) and small Cajal body RNPs. In Saccharomyces cerevisiae, H/ACA snoRNPs contain four proteins: Nhp2p (L7ae in archaea (6) and Cbf5p, also called dyskerin, in humans (7)), Nop10p, Gar1p, and a single small nucleolar RNA (snoRNA), specific to each snoRNP (8−11). Cbf5p provides the pseudouridylase activity to the complex, and the snoRNA component provides the “guide RNA” for positioning the substrate RNA for modification (8, 10, 12−15). The 3′ end of human telomerase RNA (hTR) contains an H/ACA scaRNA domain that binds the H/ACA proteins and is required for 3′ end processing, accumulation, and localization of hTR to Cajal bodies (16−19). In archaea, the assembly of H/ACA snoRNP appears to proceed by assembly of the protein components, followed by the incorporation of the H/ACA RNA (8, 20−23). In eukaryotes, the assembly and final maturation of the holoenzyme RNP are more complicated, possibly because of subcellular compartmentalization, and require accessory proteins (22, 24). Two proteins specifically found in eukaryotes, Naf1p and Shq1p, were initially identified in yeast as factors involved in the assembly of H/ACA snoRNPs (23−25). Both Shq1p and Naf1p are essential proteins, and their depletion leads to the loss of H/ACA snoRNAs (22, 24). Shq1p and Naf1p interact with the H/ACA RNP components Cbf5p and Nhp2p as shown by high throughput proteomic approaches and by directed protein interaction studies (24, 26−28). Both Naf1p and Gar1p contain a central domain that forms a six-stranded β-barrel fold and interact competitively with Cbf5p using this “core-Gar1” domain (29).

Although Shq1p was first identified in yeast (24), orthologues have been found in all eukaryotic genomes investigated, including human (22). Shq1p is not associated with the precursor or mature RNPs and is localized in the nucleoplasm (24) rather than in the nucleolus or Cajal bodies where mature H/ACA RNPs reside. It was therefore proposed that Shq1p is involved in the early biogenesis steps of H/ACA snoRNPs. However, Shq1p does not share any homology with either Naf1p or Gar1p, and its mode of action remains unclear. Based on the Saccharomyces Genome Database base annotations and domain predictions, Shq1p seems to be a modular protein with two predicted domains in its sequence (Fig. 1A). The C-terminal half contains a region of high sequence homology with other
Shq1 proteins called the Shq1 domain, but this region shows no identified folding motif. The N-terminal region of the protein has a predicted CS (named after CHORD-containing proteins and §GT1) or HSP20-like domain, which is found in a number of co-chaperones for heat shock protein 90 (Hsp90) (30–34), and hence is presumed to be an Hsp90 (Hsp82p in yeast) binding domain. The CS, HSP20-like, and p23-like domains belong to the HSP20 domain superfamily.

We have determined the x-ray crystal structure of the CS domain of Shq1p and investigated its importance for Shq1p function. The structure consists of a β-sandwich immunoglobulin light chain fold (35). Like other CS domains, the CS domain of Shq1p is primarily composed of two β-sheets (30, 31, 33, 36), but it has an additional short helix at the C terminus. We show that the Shq1p CS domain is essential for growth in S. cerevisiae and that the integrity of the CS domain is required for the function of Shq1p in vivo. Based on the structure and sequence conservation among Shq1 proteins, we investigated the effect of three single point mutations, F6A, Q10A, and K80A, on Shq1p structure and function. All three mutations destabilized the tertiary structure of the CS domain, albeit to differing extents. Incorporation of these mutations in Shq1p resulted in a temperature-sensitive growth phenotype, specific depletion of H/ACA snoRNAs, and rRNA processing defects. NMR chemical shift mapping showed no interaction between the Shq1p CS domain and Hsp82p, suggesting that the Shq1p CS domain does not have a canonical role as an Hsp82p co-chaperone protein.

**EXPERIMENTAL PROCEDURES**

*Protein Expression and Purification for Structural Studies—* CS (residues 1–101) and CS-L (residues 1–145) constructs were PCR-amplified using yeast genomic DNA as a template and cloned into pET46 LIC/Ek (Novagen). F6A, Q10A, and K80A mutant constructs of the CS domain were made by site-directed mutagenesis (Stratagene), and plasmids were transformed into *Escherichia coli* BL21 (DE3) cells for protein expression. For selenomethionine labeling, *E. coli* B834 (DE3) cells (Novagen) were used. Cells were grown either in LB medium (for unlabeled proteins) or M9 medium (for selenomethionine, 13C,15N-labeled, and 15N-labeled protein). Cultures were grown at 37 °C until an A600 of 0.6 was reached, and then the temperature was lowered to 28 °C (22 °C for the F6A and Q10A mutants) and the cells were induced by 1 mM isopropyl-1-thio-β-β-d-galactopyranoside for 16 h. Cells were harvested by centrifugation, and the pellets were resuspended in the binding buffer (30 ml; buffer A: 50 mM NaH2PO4, pH 8, 300 mM NaCl, 10 mM β-mercaptoethanol). Cells were lysed by sonication on ice and centrifuged to remove cell debris. The supernatant was applied to a nickel-nitritotriacetic acid-agarose column (5 ml), which had been pre-equilibrated with buffer A. The column was washed with buffer A, followed by buffer B (buffer A supplemented with 20 mM imidazole) and were eluted with buffer C (buffer A supplemented with 250 mM imidazole). Proteins were further purified by Superdex 75 preparative gel filtration. Yeast Hsp82p was PCR-amplified and cloned into pET30 LIC/Xa. Protein was expressed in *E. coli* at room temperature and purified over a nickel-nitritotriacetic acid-agarose column followed by gel filtration on Superdex S75 column. All proteins were >95% purified as judged by SDS-PAGE. The buffer for NMR and CD spectroscopy was 20 mM NaH2PO4, pH 7.2, 100 mM NaCl, and 2 mM dithiothreitol, 0.02% NaN3 and for crystalization was 20 mM Tris base, pH 7.2, 100 mM NaCl, 2 mM dithiothreitol, and 0.02% NaN3.

**NMR Spectroscopy—** Uniformly 13C,15N-labeled samples of CS(WT) were used for NMR assignments, and 15N-labeled samples of WT and mutant CS domains were used for other NMR experiments. NMR spectra were recorded on Bruker DRX 600-MHz and DRX 800-MHz spectrometers equipped with cryoprobes. NMR samples contained 0.3 to 0.8 mM protein. For the 1H,15N HSQC spectra, a total of 1024 complex points in t2, and 256 complex points at t1 increments were acquired. The backbone assignments for CS(WT) were obtained by standard methods using the CBCACONH/CBCANH and HNCACO/HNCO pairs of triple resonance experiments at 22 °C (37). NMR titrations were carried out by adding unlabeled purified Hsp82p to 15N-labeled CS or CS-L and recording 1H,15N TROSY-HSQC spectra. For the 1H,15N TROSY-HSQC spectra, a total of 1024 complex points in t2, and 256 complex points at t1 increments were acquired. All the titrations were carried out at 22 °C at 600 MHz. NMR data were processed using the Bruker program Xwin-NMR version 3.5 and assigned using the Sparky software program (T. D. Goddard and D. G. Kneller, SPARKY version 3, University of California, San Francisco).

**Crystallization, Data Collection, and Structure Determination—** Crystallization was carried out with a hanging drop, vapor-diffusion method by mixing equal volumes of protein and reservoir solutions (0.1 M BisTris, pH 6.0, 0.2 M ammonium sulfate, 25% w/v PEG 3350). Crystals appeared under the same conditions for both normal and selenomethionine derivatives within 24 h. Crystals grew to maximum size of 0.2 × 0.2 × 0.3 mm in 4 days and belonged to space group P212121. Unit cell dimensions and other parameters are given in Table 1.

Data were collected at the Advanced Photon Source at beamline 24-ID-C. Data were processed with the DENZOSCALEPACK package (38). The selenomethionine derivative was measured at peak wavelength of 0.97949 Å. Datasets were of high quality and showed a strong anomalous signal. Macromolecular phasing was carried out by HKL2MAP (39). The resulting phases were improved by using the DM program (40) and used for an automated model building with the Arp/Warp software (40). The resulting model of about 90% completeness was inspected and finished manually with the program Coot (41). Restrained refinement enforced by the Refmac5 software was then performed and was followed by the addition of water molecules by using Arp/Warp (42). Final iterative rounds of model building and refinement were carried out using Coot (41) and PHENIX (43). Final data collection, phasing, and refinement statistics are presented in Table 1. Most of the model has a clear electron density, with the exception of the N-terminal His tag (15 residues) and 3–4 C-terminal residues. Additionally, some solvent-exposed side chains had no interpretable electron density. These parts were omitted in the final model. The R-factor of the presented structure is 22.5%, and Rfree is 24.6%. All struc-
ture figures were drawn using PyMOL (DeLano Scientific, San Carlos, CA).

CD Spectroscopy—CD spectra of the proteins were obtained on a J-715 spectropolarimeter (Jasco J715 model). Spectra were recorded using 1-mm path length quartz cuvettes. Each spectrum was recorded as an average of four scans. For thermal denaturation experiments, spectra were monitored at 215 nm, and the sample temperature was increased at a rate of 30 °C/h.

Cloning—DNA cloning and DNA manipulations were performed as described (44). Plasmids expressing the GFP fusions in yeast were constructed by inserting DNA fragments containing the PCR-amplified SHQ1 open reading frame into pUG23 and pUG35 (45) using oligonucleotides with XbaI and EcoRI restriction sites. Constructs expressing Shq1p residues 1–493, 1–500, 9–508, 18–508, and 26–508 and point mutants F6A, Q10A, K18A, S22A, and K80A were amplified by PCR using specific oligonucleotides and inserted into pUG23. The shq1Δ knock-out strain was obtained using a PCR-based gene deletion strategy described in Ref. 47, by homologous recombination of a DNA fragment containing the coding region of TRP marker flanked by the last 40 and first 40 nucleotides of the 5′- and 3′- untranslated region, respectively, of SHQ1. The TRP cassette was co-transformed with a plasmid containing a wild-type SHQ1 gene and URA3 auxotrophy marker (pUG35-SHQ1) in a wild-type yeast strain BMA64 (ura3-3; trp1; ade2-1; leu2-3,112; his3-11,15).

Western Blots—Whole-cell lysates were prepared at 4 °C from equal A600 amounts of exponentially growing yeast cultures. Cells were resuspended in a buffer containing 1.85 M NaOH, 7.4% β-mercaptoethanol, and trichloroacetic acid at a final concentration of 5.48%. Lysates were centrifuged, the pellets resuspended, and the proteins fractionated by SDS-PAGE. Immunoblotting was performed with anti-GFP. Hexokinase (Hxk) was used as a loading control.

FIGURE 1. Deletion analysis and stability of Shq1p truncated mutants. A, domain organization of Shq1p and complementation of the shq1Δ strain by the various truncation mutants. Numbers refer to SHQ1 amino acid residues. Cells were grown in plates containing 5-fluoroacetic acid to counterselect the wild-type (wt) SHQ1 copy borne by the URA3-containing plasmid and in the absence of methionine to express Shq1p, and cell growth was assessed. B, example of growth assay used to generate the previous figure. Shown is the growth of strains expressing the indicated deletions of Shq1p on a plate containing 5-fluoroacetic acid to counterselect the wild-type SHQ1 copy borne by the URA3-containing plasmid. C, stability of truncated mutants. Cells containing the endogenous SHQ1 copy and the indicated constructs were grown on liquid medium in the absence of methionine. Proteins were expressed under the control of the Met25 promoter, which is highly induced in the absence of Met. Numbers refer to SHQ1 amino acid residues. Shq1p proteins were detected by immunoblotting with anti-GFP. Hexokinase (Hxk) was used as a loading control.
Structure and Functional Analysis of Shq1p CS Domain

RNA Analysis—Total RNA extractions and Northern blot analyses were performed as described (48). Oligonucleotides used for snoRNA probes are described in Ref. 48. Total RNA was extracted from cells incubated at 37 °C for different time intervals and hybridized against 32P-labeled oligonucleotide probes. The 35 S pre-rRNA was detected using 5′ETS and 18 S probes; the 33 S pre-rRNA was detected using 18 S probe; the 27 S pre-rRNA was detected with the ITS2 probe; and the 20 S pre-rRNA was detected using 18 S probe. The 35 S pre-rRNA was detected using 5′H11032/H11033 probes. The 35 S pre-rRNA was detected using 5′ETS and 18 S probes; the 33 S pre-rRNA was detected using 18 S probe; the 27 S pre-rRNA was detected with the ITS2 probe; and the 20 S pre-rRNA was detected using 18 S probe. Essential Functions

N-terminal deletions was designed (Fig. 1). Further deletions in the N-terminal domain resulted in protein instability (Fig. 1C), precluding us from drawing conclusions about these constructs. Overall the data obtained with the 9–508, 18–508, and 26–508 constructs show that although stably expressed, proteins lacking the first N-terminal amino acids of the Shq1p CS domain cannot functionally complement the absence of Shq1p. These data strongly suggest that the CS domain is required for Shq1p essential functions in vivo.

Structure of the Shq1p CS Domain—These results led us to carry out further functional and structural characterization of the CS domain. Based on the predicted domain boundaries and secondary structure for the CS or HSP20-like domain of Shq1p, we cloned, expressed, and purified two constructs, CS (residues 1–101) and the longer CS-L (residues 1–145) which encompasses the entire N-terminal region containing the HSP20-like and CS domain (Fig. 1A). 15N-1H two-dimensional HSQC NMR spectra show that both protein constructs contain a well folded domain (Fig. 2). The backbone amides of the CS domain constructs were assigned using CBCACONH/CBCANH and HNCA/HNCOCA sets of triple resonance NMR spectra (see “Experimental Procedures”). Comparison of the spectra of CS and CS-L reveals that all of the resonance peaks in the CS-L spectrum that are from the additional 44 C-terminal residues are in the region generally corresponding to unstructured or unfolded residues. In addition, the amide resonances from the last ~25 residues of CS-L were not visible in the 1H-15N HSQC spectra, consistent with this region being unstructured (Fig. 2). We recorded the 1H-15N HSQC of the CS-L construct under different salt, pH, and temperature values; however, we did not observe these C-terminal amide resonances under any of these conditions. Thus, the structured region of CS-L is entirely contained in CS.

Crystallization screens were set up for both CS and CS-L; however, crystals appeared only for CS. CS-L did not crystallize, possibly because of the flexible C-terminal region in the protein. The CS domain crystallized in the P21212 space group with six copies of the molecule in one asymmetric unit. However, the CS domain, as well as full-length Shq1p, purified as monomers on a gel filtration column (data not shown). The NMR line widths are also consistent with what would be expected for a monomer. Attempts to solve the structure by molecular replacement using the structure of p23 (33) as a template were not successful. Therefore, the structure was solved by the single anomalous diffraction method using a selenomethionine derivative. The final resolution of the structure was 2.4 Å (Fig. 3, A

FIGURE 2. 1H-15N HSQC spectra of the Shq1p CS domain. Overlay of 1H-15N HSQC spectra of Shq1p CS (red) and CS-L (cyan) proteins. Assignments of amide resonances of CS are indicated (K0 is a residue from the tag).

RESULTS AND DISCUSSION

Integrity of the CS Domain of Shq1p Is Required for Shq1p Essential Functions—To assess the importance of the putative CS domain of Shq1p in vivo, a series of constructs harboring N-terminal deletions was designed (Fig. 1A) and expressed as GFP fusion proteins in S. cerevisiae. The CS or alternatively named HSP20-like domain is predicted to start at the precise N terminus of Shq1p and extends approximately to residue 145 (Fig. 1A). We also expressed a series of Shq1p C-terminal deletions (Fig. 1A) as controls. Strikingly, proteins deleted of only a few amino acids from the N terminus of Shq1p (Shq1p 9–508) could not complement the SHQ1 knock-out and resulted in a lethal phenotype (Fig. 1, A and B). In contrast, C-terminal deletions of up to 17 residues did not affect cell growth (Fig. 1, A and B).

To investigate whether these phenotypes were because of instability of the mutant proteins, we monitored the expression levels of these constructs in a wild-type strain expressing these constructs in addition to the endogenous wild-type SHQ1 copy (Fig. 1C). The mutant proteins were detected using anti-GFP antibodies because these constructs were expressed as GFP fusions. Constructs expressing non-functional versions of Shq1p such as 9–508, 18–508, and 26–508 were expressed to levels similar to wild type (Fig. 1C). Further deletions in the N-terminal domain resulted in protein instability (Fig. 1C), precluding us from drawing conclusions about these constructs. Overall the data obtained with the 9–508, 18–508, and 26–508 constructs show that although stably expressed, proteins lacking the first N-terminal amino acids of the Shq1p CS domain cannot functionally complement the absence of Shq1p. These data strongly suggest that the CS domain is required for Shq1p essential functions in vivo.
We did not observe electron density for the N-terminal His tag (first 15 residues) and the last four residues at the C terminus, and these were therefore omitted in the final refinement and structure.

The overall fold of Shq1p CS is a compact β-sandwich consisting of two β-sheets, confirming that this region of Shq1p has a CS domain as predicted (Fig. 3A) (30, 33). The larger β-sheet is formed by β1 (residues 6–10), β2 (residues 14–20), β6 (residues 64–68), and β7 (residues 72–79) strands, and the other β-sheet includes β3 (residues 32–36), β4 (residues 39–44), and β5 (residues 47–52) strands. The C-terminal residues 93–97 form a short helix α1, which is usually not present in CS domains (Fig. 3A).

Sequence alignment of Shq1 from ~55 different species showed that the CS domain is conserved throughout many eukaryotic orthologues (22, 24) (Pfam data base). Fig. 3C shows the sequence alignment of eight Shq1 proteins from different species. Among the highly conserved residues, Lys-80 is absolutely conserved, whereas Phe-6, Gln-10, Phe-87, Leu-96, and Leu-97 are the most highly conserved residues (>90%). Of these, based on their location and interactions in the structure, F6A, Q10A, and K80A appeared to be important for the stability of the folded protein. Residues Phe-6 and Gln-10 are located in the β1 strand, which is part of the first β-sheet. The aromatic ring of Phe-6 is located in the interior of the protein and makes a hydrophobic interaction with the side chain of Leu-97, contributing to the folding of the domain. Interestingly, Phe-6 was also found as one of the muta-
tions in a screen for temperature-sensitive mutants using a random mutagenesis approach. The Gln-10 side chain O/H9280 and N/H9280 make hydrogen bonds with the backbone amide N and carbonyl O of Phe-87, which is also a highly conserved residue. The side chain of the conserved Lys-80 is almost completely buried inside the hydrophobic interior of the protein, and its backbone amide N makes a hydrogen bond with the backbone carbonyl oxygen of Glu-13. Based on their location in the structure (Fig. 4F) and strong sequence conservation, we characterized the effect of alanine substitutions of these residues on the CS domain structure and stability as described below.

Biophysical Characterization of CS Domain Mutants—Expression of the wild-type CS domain in E. coli was efficient, yielding ~10 mg of purified protein per liter of LB or minimal media. However, the expression levels of the CS(F6A) and CS(Q10A) mutants were >10-fold lower than the wild-type CS domain, and (K80A) did not express in all of our expression attempts. We reasoned that CS(K80A) may have been unfolded and degraded, and therefore there was no detectable expression of the full-length domain in E. coli.

To assess the effect of the amino acid substitutions on the CS domain structure, 1H-15N HSQC spectra were recorded as a function of temperature (15, 22, 25, 30, and 37 °C) for CS(F6A) and CS(Q10A) and compared with the spectra of CS(WT). CS(WT) was stable and showed spectra of a folded protein at all the investigated temperatures. At temperatures up to 25 °C, CS(F6A) exhibited 1H-15N HSQC spectra of a folded protein, although many peaks were shifted compared with the wild-type CS, because of the substitution of Ala for the aromatic Phe (Fig. 4A). However, at 30 °C many of the resonance peaks were broadened and/or shifted into the region of the spectrum typical for unfolded proteins or molten globules (~8.3 ppm, in 1H dimension). At 37 °C most of the resonances disappeared and were not recovered upon reacquisition of data at 25 °C, indicating that the protein was irreversibly denatured (Fig. 4B). The 1H-15N HSQC spectra of CS(Q10A) show that the domain is already partially unfolded at 15 °C, and it remains partially folded at 30 °C and above, the peaks in the 1H-15N HSQC of CS(Q10A) disappeared because of denaturation (Fig. 4D). Analysis of the spectra indicates that for both CS(F6A) and CS(Q10A), the resonances that do not show chemical shift changes under conditions where the protein is still at least partially folded generally arise from residues far from the mutation site in the three-dimensional structure (data not shown).

We also checked the integrity and stability of the wild-type and mutant CS domains using CD spectroscopy. Because both wild-type and mutant proteins showed spectra typical for β-sheet protein, we chose 215 nm (a characteristic minima for the β-sheet) to monitor the change in CD signal as a function of temperature (Fig. 4E). The melting temperature for the wild-type CS domain determined by this method was about 60 °C, whereas the melting temperatures of F6A and Q10A protein

FIGURE 4. 1H-15N HSQC and CD spectra of Shq1p CS domain mutants. A–D, 600-MHz 1H-15N HSQC NMR spectra of CS(F6A) (A and B) and CS(Q10A) (C and D) at 25 °C (A and C) and 37 °C (B and D). Spectra of CS(F6A) and CS(Q10A) are shown in cyan and are overlaid on CS(WT) in red for comparison. E, thermal stability of CS (WT), CS(F6A), and CS(Q10A) monitored by CD spectroscopy. Both CS(F6A) and CS(Q10A) are less stable than CS(WT). F, shown is the ribbon model of the CS(WT) structure with side chains of residues Phe-6, Gln-10, and Lys-80 that were mutated in this study.

4 F. A. Gonzales and G. Chanfreau, unpublished results.
were 44 and 45 °C, respectively (Fig. 4E). In the case of CS(Q10A), an early melting transition was observed in the 15–30 °C range (Fig. 4E), suggesting that at least one region of the protein is undergoing a structural transition even at room temperature. These findings agree with our NMR results that show partial unfolding of CS(Q10A) at a lower temperature than for CS(F6A).

**Point Mutations within the Shq1 CS Domain Destabilize Shq1p in Vivo**—The NMR and CD spectroscopy analyses of the Shq1p CS domain mutants showed that the selected point mutations significantly destabilize the fold of this domain in vitro, and this destabilization is more severe for CS(Q10A) than for CS(F6A). Based on these results, we predicted that these mutations would also destabilize Shq1p in vivo and might confer temperature-sensitive phenotypes if the CS domain contributes to Shq1p function in vivo, as predicted from the domain deletion analysis (Fig. 1, A and B). To test this hypothesis and investigate the effect of these mutations in vivo, we introduced the F6A, Q10A, and K80A mutations into a plasmid expressing Shq1p as a GFP fusion protein, and we transformed the plasmid into a strain where the SHQ1 chromosomal copy was disrupted and covered by a URA plasmid containing a wild-type SHQ1 copy. After selection of the transformants, the URA plasmids carrying the wild-type SHQ1 copy were counter-selected by streaking strains on a medium containing 5-fluoroorotic acid (49), and strain viability was assessed by growth on plates at 25 and 37 °C (Fig. 5, A and C). Shq1p(F6A) and Shq1p(Q10A) strains were viable at 25 °C but exhibited a growth defect at 37 °C. The Shq1p(K80A) strain exhibited a strong growth defect at 25 °C and loss of viability at 37 °C. These growth defects were confirmed by growth curves obtained from liquid cultures (Fig. 5, B and D). All mutant strains exhibited longer generation times or growth arrest at 37 °C, and the Shq1p(K80A) showed a slow growth phenotype even at 25 °C. To further investigate the molecular basis of the growth phenotypes observed for these mutants, we monitored the levels of the Shq1p-GFP fusion protein expressed from these constructs. Although all the mutant proteins showed normal expression levels at 25 °C, their levels were dramatically reduced at 37 °C (Fig. 5E), in contrast to the wild type that was unaffected. These results indicate that destabilizing the CS domain in the context of the full-length protein likely leads to degradation of Shq1p in vivo.

**Point Mutations in the CS Domain Result in H/ACA snoRNA Depletion and Defects in Pre-rRNA Processing**—We further investigated the effect of the Shq1p CS domain mutations on Shq1p function in vivo by analyzing H/ACA snoRNA accumulation in the CS domain mutants grown at 25 °C or shifted to 37 °C. Although H/ACA snoRNA levels were unaffected by shifting the wild-type strain to 37 °C, all three strains expressing Shq1p CS domain mutants exhibited lower H/ACA snoRNA levels at the elevated temperature (Fig. 6A). Interestingly, the level of depletion of H/ACA snoRNAs for the mutants correlated well with the growth defects and with the structural instability monitored by NMR and CD spectroscopy and protein expression levels in E. coli. For example, the Shq1p(F6A) strain showed the weakest growth defect in vivo (Fig. 5), the highest stability of all CS mutants in vitro (Fig. 4, A, B, and E), and the lowest level of H/ACA snoRNA depletion of the Shq1p mutant strains in vivo (Fig. 6A). The Shq1p(K80A) strain, which shows a significant growth defect at 25 °C (Fig. 5, B and C), exhibited a strong snoRNA depletion phenotype at this temperature (Fig. 6A) and could not be expressed in E. coli.

Importantly, although the snoRNA depletion observed for all mutants at 37 °C is likely due to decreased Shq1p levels triggered by the thermal instability of the mutant proteins, the depletion of H/ACA snoRNAs observed in the Shq1p(K80A) and Q10A strains at 25 °C cannot be attributed to protein depletion, because protein levels were normal for these mutants at this temperature (Fig. 5E). This result further demonstrates that the CS domain is required for Shq1p function in vivo, because mutations that do not result in degradation of the protein at 25 °C generate a growth defect and an H/ACA snoRNA depletion phenotype. Finally, the levels of the C/D snoRNA snr190 were unaffected by the Shq1p CS domain mutations, consistent with previous observations that Shq1p functions exclusively in H/ACA snoRNP assembly (24) (Fig. 6A).

Because the snr10 and snr30 H/ACA snoRNAs have been shown to participate in pre-ribosomal RNA (rRNA) processing (50, 51), we predicted that the depletion of these snoRNAs caused by the Shq1p CS domain mutations (Fig. 6A) would generate a delay or a defect in pre-rRNA processing in the
Shq1p mutant strains, as shown previously for Shq1p depletion (24). In agreement with this prediction, we observed a decrease in the level of the 20 S and 27 S ribosomal RNAs processing intermediates after a shift of the Shq1p CS domain mutant strains to 37 °C (Fig. 6, B and C), although the wild-type strain was largely unaffected. Because the onset of the rRNA processing defects observed in the Shq1p CS domain mutant strains occurred concomitantly to, or after the H/ACA snoRNA depletion, we conclude that this rRNA processing defect is likely due to H/ACA snoRNA depletion caused by the CS domain mutations, rather than by a direct involvement of Shq1p in pre-rRNA processing, as shown previously for Shq1p depletion (24).

Shq1p CS Domain Does Not Interact with Yeast Hsp90 in Vitro—Hsp90 (yeast Hsp82p) is a general molecular chaperone responsible for structural maintenance and proper folding and regulation of specific target proteins involved in cell cycle control and signal transduction (32, 52). Several co-chaperones are known to bind and modulate the chaperone cycle of Hsp90. The CS/Hsp20-like domain is recognized as an Hsp90-binding module, as these domains in several co-chaperones have been shown to interact directly with Hsp90 both in vivo and in vitro (30, 33). Recently, Hsp90 was implicated in the biogenesis of the L7Ae family of RNPs, including box C/D RNPs (53). Both Hsp90 and one of its co-chaperone proteins p23, which is structurally similar to the CS domain (hence the CS domain is also called as p23-like domain), have also been shown to bind to hTERT and influence its assembly process with hTR (54, 55). Hsp82p was also reported to promote both the DNA binding and nucleotide extension properties of telomerase in yeast (56). We therefore investigated whether the Shq1p CS domain interacts with yeast Hsp90 (Hsp82p) by NMR, using chemical shift perturbation experiments. The amide chemical shifts of Shq1p CS domain in 1H-15N HSQC spectra were monitored as a function of added unlabeled Hsp82p. No changes were observed in the 1H-15N HSQC spectra of Shq1p CS domain even with a 3-fold excess of Hsp82p, indicating that there is no interaction between the two proteins under the NMR conditions (Fig. 7). We also checked the interaction between Shq1p CS-L and Hsp82p, because a region near the C terminus of Sba1 (yeast p23) was found to interact with Hsp82p (31, 36) (discussed below). Again, no spectral changes were observed upon addition of Hsp82p to CS-L, indicating the lack of binding to Hsp82p by the CS domain is not because of the absence of the extra 44 amino acids in the CS-L (HSP20-like) domain (data not shown). The CS (p23-like) domain/ Hsp90 interaction is ATP-dependent in vivo (33), although both p23 and Sgt1 CS domain can also interact with Hsp90 in...
the absence of ATP in vitro (30, 31, 33). To check whether ATP was required for interaction between Shq1p CS domain and Hsp82p, we also performed the NMR titration in the presence of ATP. Addition of ATP did not result in formation of an Shq1p CS domain-Hsp82p complex (data not shown). In contrast to these results, binding between Hsp90/Hsp82p and the p23, human Sgt1, and plant (*Arabidopsis thaliana*) Sgt1a CS domains, without and with ATP, has been detected using NMR chemical shift perturbation studies even at sub-stoichiometric ratios of HSP90/82 to CS domain (30, 57).

We compared the structure of the Shq1p CS domain with those of three proteins, Sgt1 CS domain, p23, and Sba1, which have been shown to interact with Hsp90 using NMR or crystallography and other biochemical methods (30, 31, 33, 36). Sgt1 is a subunit of the kinetochore and SCF (Skp1-Cul1-F-box) ubiquitin ligase complexes (58, 59), and p23 is a highly conserved protein that acts as a co-chaperone for Hsp90 and modulates its activity (33, 36, 60). Sequence alignment of the Shq1p CS domain, p23, human Sgt1 CS domain, and Sba1 (Fig. 8A) showed low homology (overall homology <15%, identity <2%), but all have a similar tertiary fold (Fig. 8B). However, there are significant differences among all of the proteins in the lengths of the β-strands, the loops, and the C-terminal region. The CS domain of Shq1p has a small α-helix in the C-terminal part that is replaced by a short β-strand in Sba1 and p23 and no structural element in Sgt1 CS domain (30, 31, 33, 36) (Fig. 8B). In Sba1 the lengths of the β-strands are shorter in comparison with the Shq1p CS domain (Fig. 8B).

The interactions between p23, Sgt1 CS domain, and plant Sgt1a CS domain and Hsp90 have been studied by NMR (30, 31). Chemical shift mapping of resonances that shifted or showed line broadening in the presence of Hsp90 were reported for p23 and a homology model of plant Sgt1a (30, 57). A more detailed interaction surface has been presented in the co-crystal structure of the complex of Hsp90 in an ATP-bound closed conformation with Sba1 (36). All of these studies localize the binding surface for Hsp90 on the CS domains to the face of the protein containing the β1β2β6β7 sheet. Fig. 8A shows the residues of the p23 (blue box) and Sba1 (cyan box) that were shown to be involved in the complex interactions.
formulation with Hsp90. In the crystal structure of the Sba1-Hsp82p-ATP complex, the Sba1 interacts with Hsp82p at three distinct surface patches on the β1β2β6β7 sheet. The Hsp90-interacting surface of the p23 and homology-modeled plant Sgt1 CS domain, identified by NMR chemical shift mapping, only partially overlaps two of these patches. Furthermore, based on an Hsp90-Nter-Sgt1-CS crystal structure and modeling of an Hsp90-Sgt1-CS-Sba1 complex, it has very recently been proposed that Sba1 (p23) and the Sgt1a CS domain bind at different sites on Hsp90 (61, 62). Although it is therefore difficult to identify specific differences in residues on the β1β2β6β7 surface of the CS domain that may account for the absence of Hsp90 binding to the Shq1p CS domain, comparison of the surface charge distribution of Sba1, p23, and Sgt1 CS domains with Shq1p CS reveals a significant difference (Fig. 8C). The Sba1, p23, and Sgt1 CS domains all have a large basic surface patch on the β1β2β6β7 face of the structure (30). The Shq1p CS domain does not have such an extensive basic region (Fig. 8C), which might explain the lack of binding to Hsp82.

We also checked the levels of Shq1p and snoRNAs in Hsp82p deletion strains and did not find any difference in their levels (data not shown). Interestingly, single alanine substitutions in CS surface residues Lys-18 and Ser-22 did not show any phenotypic differences (data not shown). The equivalent residues in the Sba1-Hsp82p-ATP complex are in the binding interface. Based on these observations it appears unlikely that the Shq1p CS domain binds to and functions as a co-chaperone for Hsp90. However, we cannot rule out the possibility that other regions of Shq1 or accessory proteins may interact with Hsp90.

Although CS domains generally interact with Hsp90, the Hsp20 superfamily β-sandwich fold is found in other proteins such as small heat shock proteins (sHSPs), e.g. MJHSP16.5 from *Methanococcus jannaschii* and HSP16.9B from wheat. These proteins act as molecular chaperones but do not interact with the Hsp90 (30, 63). sHSPs are involved in a variety of cellular functions, which include a role in the process of thermo-tolerance and maintenance of protein homeostasis in the cell. They form multimeric complexes with folding intermediates of substrate proteins (63–66). Hence, it is possible that Shq1p has a chaperone activity independent of Hsp90 that is required for the proper folding and assembly of H/ACA RNP components.

**Summary**—In this study we determined the crystal structure of the Shq1p CS domain. Based on the structure, we addressed two questions as follows: first, whether the CS domain is important for Shq1p function and has a role in H/ACA snoRNP biogenesis; and second, whether it interacts with the Hsp90 molecular chaperone like the CS domains of the co-chaperones p23, Sba1, and Sgt1. Our results show that the CS domain is required for the structural integrity and functions of Shq1p in vivo. There is a clear correlation between the structural properties and thermal stabilities of the isolated CS domains (WT and mutants) determined in vitro and the functional properties of these mutants observed in vivo. Several point mutations that destabilize the CS domain in vitro, as assessed by NMR and CD spectroscopy, generate growth defects, H/ACA snoRNA depletion phenotypes, and defects in rRNA processing, even in conditions when mutant Shq1p protein levels are normal (Q10A and K80A). The Shq1p CS domain has the same fold as the CS domains of Hsp90 co-chaperones Sgt1, p23, and Sgt1. However, there are differences in the structure of the Shq1p CS domain in comparison with CS domains that interact with the Hsp90 molecular chaperone family, which may account for its lack of direct interaction with Hsp82.

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Structure and Functional Analysis of Shq1 CS Domain

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