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Identification of Essential Residues in the Type II Hsp40 Sis1 That Function in Polypeptide Binding*

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Sis1 is an essential yeast Type II Hsp40 protein that assists cytosolic Hsp70 Ssa1 in the facilitation of processes that include translation initiation, the prevention of protein aggregation, and proteasomal protein degradation. An essential function of Sis1 and other Hsp40 proteins is the binding and delivery of non-native polypeptides to Hsp70. How Hsp40s function as molecular chaperones is unknown. The crystal structure of a Sis1 fragment that retains peptide-binding activity suggests that Type II Hsp40s utilize hydrophobic residues located in a solvent-exposed patch on carboxyl-terminal domain I to bind non-native polypeptides. To test this model, amino acid residues Val-184, Leu-186, Lys-199, Phe-201, Ile-203, and Phe-251, which form a depression in carboxyl-terminal domain I, were mutated, and the ability of Sis1 mutants to support cell viability and function as molecular chaperones was examined. We report that Lys-199, Phe-201, and Phe-251 are essential for cell viability and required for Sis1 polypeptide binding activity. Sis1 I203T could support normal cell growth, but when purified it exhibited severe defects in chaperone function. These data identify essential residues in Sis1 that function in polypeptide binding and help define the nature of the polypeptide-binding site in Type II Hsp40 proteins.

Hsp40s represent a structurally diverse family of co-chaperones that function with Hsp70 to facilitate cellular processes that include protein folding, the suppression of protein aggregation, endocytosis, protein translocation across membranes, signal transduction, DNA replication, protein degradation, and prion propagation (1–4). Hsp70 facilitates these processes by utilizing energy derived from ATP hydrolysis to bind and release regions of proteins that exhibit aspects of non-native structure (5–7). Hsp40s function by regulating the Hsp70 ATP hydrolytic cycle (8, 9) and by acting as molecular chaperones that bind and target non-native proteins to the peptide-binding site of Hsp70 (10, 11). To regulate Hsp70 ATPase activity Hsp40 proteins utilize a conserved region, which was identified in *Escherichia coli* DnaJ and is termed the J-domain (12, 13). The J-domain, found in all Hsp40s, is around 70 amino acids in length and contains a conserved HPD tripeptide that is the signature motif of this protein family (14). The NMR structure of the J-domain shows it to contain four α-helical regions with the HPD motif being located in a loop that connects Helix II and Helix III (15–17). How the J-domain regulates Hsp70 ATPase activity is not entirely clear, but a surface formed by helix II and the HPD motif is proposed to bind a cleft at the base of the Hsp70 ATPase domain and thereby stimulates ATP hydrolysis (18–20). Energy derived from ATP hydrolysis then drives a conformational change in Hsp70 that is proposed to involve the closure of a lid structure that covers the peptide-binding groove and stabilizes Hsp70-peptide complexes (6, 21).

How Hsp40s function as molecular chaperones to bind and deliver non-native proteins to Hsp70 is not well established (23). The study of the mechanism for Hsp40 chaperone function is complicated by the fact that Type I, II, and III Hsp40s are not functionally equivalent (24–28). Biochemical studies with purified Type I Hsp40s such as *E. coli* DnaJ, human Hdj-2, and yeast Ydj-1 demonstrate that these proteins function as chaperones independent of Hsp70 to suppress protein aggregation (11, 29). On the other hand, Type II Hsp40s such as human Hdj-1 and yeast Sis1 appear to be less efficient as chaperones and need to act with Hsp70 to suppress protein aggregation (27, 30). Type III Hsp40s do not appear capable of suppressing protein aggregation or facilitating protein folding and, therefore, may not function as molecular chaperones (2). Differences in the structures of Type I, II, and III Hsp40s appear to account for the differences in chaperone activity exhibited by these co-chaperone proteins. Type I Hsp40s are modeled after DnaJ and contain a J-domain, a Gly and Phe (G/F)-rich region, a zinc finger-like domain, and a conserved carboxyl-terminal domain (CTD).[1] Biochemical and genetic studies suggest that Type I Hsp40s utilize the zinc finger-like region and portions of CTD to bind non-native proteins (31–33). Type II Hsp40s contain the J-domain, G/F-rich region, and the CTD but lack the zinc finger-like region, which is replaced in part by a Gly and Met (G/M)-rich region (2). Biochemical and genetic studies suggest that the G/F region and portions of the conserved carboxyl terminus enable Type II Hsp40s to function as chaperones (4, 27, 34). Type III Hsp40s contain the J-domain and other specialized structures that enable them to bind specific proteins, nucleic acids, and insert into intracellular membranes (2). Thus, Hsp40s have evolved to contain different types of polypeptide-binding domains, and this structural divergence enables them to direct Hsp70 to bind a broad range of substrates.

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[1] The abbreviations used are: CTD, carboxyl-terminal domain; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; BSA, bovine serum albumin; LA, α-lactalbumin; R-LA, reduced lactalbumin; D-Luc, denatured luciferase; GRASP, graphical representation and analysis of structural properties.

To investigate the mechanism for the chaperone function of Type II Hsp40s, we utilized the yeast Sis1 protein as a model protein (25). Sis1 is an essential 352-amino acid residue protein that functions in the cytosol with members of the Hsp70 Ssa family (27, 35). Biochemical studies show that the polypeptide binding activity of Sis1 is retained by a fragment of the protein that contains residues 171–352 (Sis1-(171–352)) (27). Consistent with these data, genetic studies have demonstrated that the CTD of Sis1 carries out functions that are essential to support cell viability (36). However, the mechanism by which Sis1 binds and delivers non-native polypeptides to Hsp70 is not clear.

Insight into the nature of the Sis1 peptide-binding site was provided by the crystal structure of Sis1-(171–352), which reveals that CTD of Sis1 forms a crystallographic homodimer that has a wishbone-like structure (37). Sis1-(171–352) monomers have an elongated shape and contain two barrel-like domains, CTDI and CTDII, and a C-terminal dimerization motif that correspond to residues 180–255, 260–329, and 330–352, respectively (37). Deletion of the dimerization domain of Sis1 reduces its ability to help Hsp70 refold luciferase (37), but monomeric Sis1 can still support the growth of yeast (36). Thus, Sis1 can carry out its essential functions as a monomer, and contrary to a previous suggestion (23), the dimerization domain is not likely to play a direct role in polypeptide binding.

To bind non-native polypeptides, chaperone proteins typically utilize regions enriched in solvent-exposed hydrophobic amino acid side chains (38). Analysis of the Sis1-(171–352) structure revealed the existence of a hydrophobic patch of amino acids located on the surface of domain I, which was predicted to participate in Sis1 chaperone function (37). To test this model, we carried out a mutational analysis of residues present in the hydrophobic patch in CTDI of Sis1. The results reported herein demonstrate that highly conserved residues within CTDI are essential for cell viability and are required for Sis1 to bind non-native polypeptides.

MATERIALS AND METHODS

Subcloning and Site-directed Mutagenesis of Sis1—To produce a vector to drive the overexpression of Sis1 in *E. coli*, the coding sequence of *Sis1* was amplified from yeast genomic DNA by polymerase chain reaction (PCR) with the 5′-primer, SIS-N (5′-ACAGAACTAACCATG-GTCAAGGAGACAAACT T-3′), and the 3′-prime primer, SIS-C (5′-TGCTTAGGATCCCTATTAAAAATTTTCATCTAT AGC-3′). This PCR product was then cloned into the *Nde*I and *Bam*HI sites present in the polylinker of the *E. coli* expression vector pET9a (39) to generate pET9a*Sis1*.

To express Sis1 from a plasmid in yeast under the control of its own promoter the primers SIS-UN (5′-ATGACCATCGATCATCCATCTGT-TGTCCTGTGAAAAGA-3′) and SIS-C were utilized to generate a PCR fragment that contained bases that were –772 to 1056 from the *Sis1* start codon (25). This PCR fragment contains both the *Sis1* promoter and open reading frame and was subcloned into the *Spe*I and *Bam*HI sites present in the polylinkers of the centromeric yeast expression plasmids pRS314 and pRS315 (40) to generate pRS314*Sis1* and pRS315*Sis1*.

To construct the Sis1 point mutants characterized in this study (see Fig. 2), a 4-primer PCR-based mutagenic protocol was utilized (41). Briefly, the primers, SIS1-N and SIS1-C were employed in combination with a set of internally overlapping mutagenic primers to generate PCR products that contained a single point mutation in *Sis1*. The mutated *Sis1* PCR products were then digested with *Stu*I and *Bam*HI to generate a DNA fragment that contained bases 148–1056 of *Sis1*. pET9a*SIS1*, pRS314*Sis1,* and pRS315*SIS1* were then digested with *Stu*I and *Bam*HI, and the mutated and digested Sis1 PCR fragments were utilized to replace the region of the wild-type *Sis1* open reading frame present in these plasmids that corresponded to bases 148 to 1056.

Purification of Hsp70 Ssa1 and Sis1—Yeast Hsp70 Ssa1 was purified from yeast strain MW141 (42) grown in YP medium containing 2% galactose to an A_{600} of 3. Hsp70 Ssa1 was then purified using ATP-agarose, ion exchange, and hydroxyapatite chromatography as described previously (9). Wild-type and mutant Sis1 were overexpressed in *E. coli* BL21(DE3)pLys by induction with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside followed by growth for 3 h at 30 °C. Purification of Sis1 was then carried out by ion exchange and hydroxyapatite chromatography as described previously (27). Purified proteins were stored on ice or at −80 °C prior to use.

Assays for Sis1 Protein Folding and ATPase Regulatory Activity—The ability of Sis1 to cooperate with Hsp70 Ssa1 to facilitate the refolding of chemically denatured luciferase was monitored as described previously (27). The ability of Sis1 to stimulate the ATPase activity of Hsp70 Ssa1 was monitored by thin layer chromatography with polyethyleneimine-cellulose plates as previously described (9).

Limited Proteolysis of Purified Sis1—Purified Sis1 (0.3 mg/ml) was incubated at 30 °C for 1 h. in 30 ml of buffer (10 mM Hepes, pH 7.4, 150 mM KCl, and 5 mM DTT) that was supplemented with proteinase K (0.01–1.0 mg/ml). Digestions were terminated by the addition of 0.5 mM phenylmethylsulfonyl fluoride, and samples were immediately added to SDS-PAGE sample buffer and run out on 12.5% SDS-PAGE. Previous studies have demonstrated that the proteolytic products liberated from Sis1 to be a 21-kDa band that corresponds to residues 171–352 and a pair of 7–9-kDa bands that represent fragments containing the J-domain (27).

Assay for the Binding of Sis1 to Non-native Polypeptides—To compare the peptide binding activity of Sis1 and the Sis1 mutants, a binding assay representing a modified version of the enzyme-linked immunosorbent assay (ELISA) method for detecting complex formation between DnaJ and its substrates was established (43). The assay is based on the ability of purified Sis1 to bind non-native proteins immobilized on the surface microtiter plate wells with the retained protein being detected via ELISA. To immobilize firefly luciferase in the wells of microtiter plates, it was first chemically denatured by incubation at 5 mg/ml in 3 M guanidine HCl, 25 mM Hepes, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, and 5 mM DTT for 1 h at room temperature. Then, 0.2 mg of denatured luciferase-made 0.1 M $NaHCO_3$ (pH 8.6) was added to wells and incubated for 30 min at 25 °C. Dot blot analysis demonstrated that under these conditions more than 90% of the added luciferase was retained in the wells. When the immobilization reaction was complete, wells were washed twice with PBS (50 mM phosphate, pH 7.4, 150 mM NaCl) and then blocked with 150 μl of 0.5% bovine serum albumin in PBS for 30 min. Wells were then washed three times with PBST (PBS containing 0.05% Tween 20). Sis1 or Sis1 mutants were then added to the wells in PBST supplemented with 0.2% BSA (PBST/BSA). After a 1-h incubation at 25 °C, the wells were washed five times with PBST. α-Sis1 rabbit polyclonal sera in 50 ml of PBST/BSA was added to the wells at a 1:5000 dilution and incubated for 1 h at 25 °C. Wells were washed five times, and then goat anti-rabbit horseradish peroxidase secondary antibody (1:5000 dilution in 50 ml PBST/BSA) was added, and incubations were carried out for 45 min. After five washes, peroxidase substrate solution was added to each well, and color formation was determined using microplate reader (Bio-Rad) set at 415 nm. Peroxidase substrate solution was prepared immediately prior to use by mixing 36 μl of 30% H_2O_2 and 21 ml of filtered ABTS stock solution (22 mg of ABTS/100 ml of 50 mM sodium citrate, pH 4.0).

Results from control experiments demonstrated that Sis1 could be detected via ELISA over a 0.1 to 100 ng range of concentrations. In addition, we demonstrated via Western blot that all of the Sis1 mutants exhibited the same immunoreactivity to α-Sis1 as to Sis1.

In experiments where reduced α-lactalbumin (LA) was utilized as the immobilized substrate of Sis1 the following protocol was employed to generate this substrate. Bovine α-LA (type III, Ca^{2+}-depleted; Sigma) at 5 mg/ml was incubated in 10 mM DTT, 0.1 M Tris (pH 8.7), 0.2 M KCl, and 1 mM EDTA for 15 min at °C. Then 0.4 μg of reduced LA (R-LA) was added to the wells of microtiter plates in 0.1 M $NaHCO_3$ (pH 8.6) supplemented with 5 mM DTT in a volume of 50 μl. Complex formation between immobilized R-LA and Sis1 was then monitored as described above, except R-LA was maintained in its reduced stated by the addition of 2 mM DTT to all reaction mixtures.

Assay for the Ability of Sis1 Mutants to Support the Growth of Yeast—The *in vivo* function of the *Sis1* CTDI mutants was analyzed by determining whether they could support the growth of a *sis1*Δ strain (MATa *ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 ssd1-D2 can1-100 sis1*::*His3*; (25) or a *sis1*Δ::*ydj1*Δ strain (JJ1146;MATa *trp1-1 ura3-1 leu2-3,112 his3-11,15 ade2-1 can1-100 met2-D1 lys2-D2 ydj1*::*His3 sis1*::*Leu2* (36). The viability of these respective strains was supported by *Sis1* supplied on the low copy *Ura3* plasmid pRS316 (40). To swap wild-type *Sis1* for its mutant forms the plasmid shuffle technique was utilized (44). The *sis1*Δ strain was transformed with wild-type or mutant *Sis1* that was supplied on a low copy *Leu2* plasmid pRS315 (40). The *sis1*Δ::*ydj1*Δ strain was transformed with wild-type or mutant *SIS1*

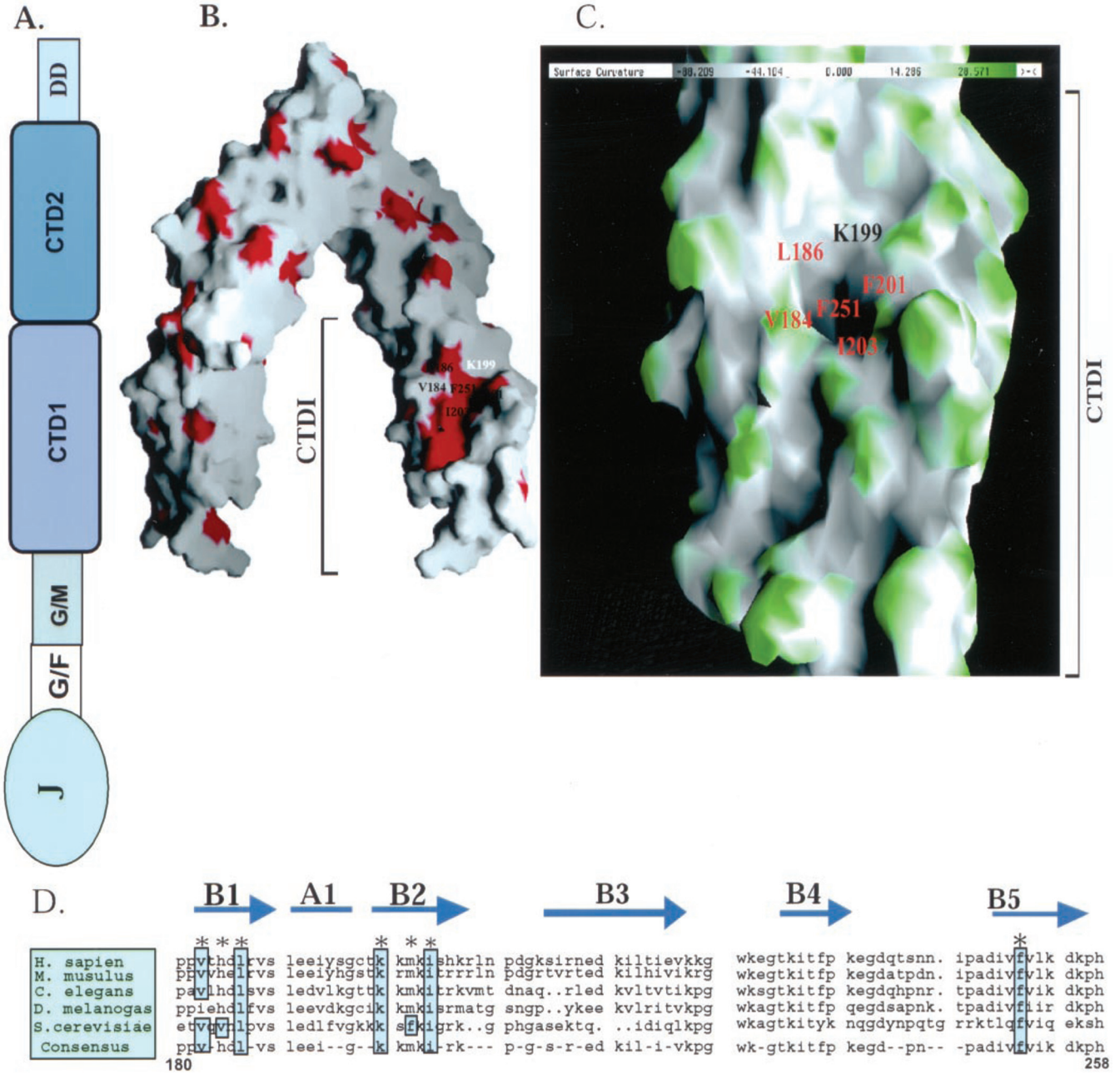

FIG. 1. **The domain structure of Sis1.** *A*, schematic representation of the Sis1 domain structure. The subdomains of Sis1 are labeled as follows: *J*, J-domain; *G/F*, Gly/Phe-rich region; *G/M*, Gly/Met-rich region; *CTD1* and *CTD2*, carboxyl-terminal domains 1 and 2; *DD*, dimerization domain. *B*, GRASP representation of solvent-exposed hydrophobic residues on the surface of the Sis1-(171–352) crystal structure. *Red* denotes hydrophobic regions that are formed by carbon atoms in the side chains of Ala, Ile, Leu, Met, Phe, Pro, and Val. *C*, GRASP representation of the contours within the hydrophobic patch located on the surface of CTD1. The colors *green, gray*, and *white* denote convex, concave, and planar surfaces, respectively. The *labels* denote the solvent-exposed hydrophobic amino acid residues that are present within the patch in single-letter code. *D*, sequence alignment of CTD1 from Type II Hsp40 proteins from five different genera of organisms. The Type II Hsp40 from *Saccharomyces cerevisiae* corresponds to residues 180–258 from Sis1. *Asterisks* highlight the position and conservation of the solvent-exposed residues depicted in *C*. *Arrows* and *bars* labeled *B1–5* and *A1*, respectively, mark the β-strands and α-helical region within CTD1.

that was supplied on a low copy *Trp1* plasmid pR314 (40). To counter select for the *Sis1* present on the *Ura* plasmid transformants were grown on media that contained 5-fluoroorotic acid (44). Strains were grown at 25 °C for 7 days, and the plates were then photographed.

Western Blot Analysis of Sis1 Expression—The steady state expression levels of *Sis1* mutants were analyzed by Western blot of yeast extracts with a rabbit polyclonal Sis1 antibody. Freshly selected strains were grown in selective media to an A_{600} of 2. Yeast cells were fixed with 5% trichloroacetic acid for 5 min, and then cell pellets were twice washed with 80% acetone and resuspended in SDS-PAGE sample buffer. Lysate proteins (5 mg) were resolved on 12% SDS-PAGE and then transferred to nitrocellulose membranes.

To examine the influence of wild-type *Sis1* expression on the steady state level of the respective *Sis1* mutants, a *sis1*Δ strain that harbored *Sis1-His*$_6$ on low copy pRS316 was generated. This strain was then transformed with wild-type and mutant forms of *Sis1* on low copy pRS315 and transformants were selected on synthetic medium that was devoid of leucine and uracil. When extracts of these strains were prepared and run on 15% gels the Sis1-His$_6$ protein migrated with a slower mobility than Sis1. This allowed for the visualization of the expression levels of non-tagged Sis1 mutants and Sis1-His$_6$ in Western blots of cell extracts.

RESULTS

Identification of Solvent-exposed Hydrophobic Residues in the Sis1 CTD—To identify regions in Sis1-(171–352) that might function in peptide-binding, GRASP analysis was utilized to

probe the structure of this fragment for solvent-exposed hydrophobic residues (Fig. 1, *A* and *B*) and contours (Fig. 1*C*). This analysis identified an unoccupied solvent-exposed patch of hydrophobic amino acid residues located on CTDI of Sis1 monomers. This patch represented the largest solvent-exposed hydrophobic region on the surface of Sis1 and is formed by residues that are contributed by β-strands 1, 2, and 5 (Fig. 1, *C* and *D*). A distinguishing feature of this patch is that it contains a 5-Å deep depression in which the solvent-exposed surface is lined by highly conserved residues that are both aliphatic and aromatic in nature (Fig. 1*C*). Sequence alignment of CTDI from Sis1 with similar regions from other Type II Hsp40 proteins demonstrates that residues Leu-186, Lys-199, Ile-203, and Phe-251 are 100% conserved (Fig. 1*D*). Whereas Val-184 and Phe-201 are found in only 20% of the Type II Hsp40s analyzed. However, in 80% of the cases a methionine residue has conservatively replaced Phe-201. Thus, CTDI of Sis1 contains a patch of solvent-exposed residues in which lies a depression that is primarily lined by conserved hydrophobic amino acids having the potential to be involved in substrate binding.

Sis1 CTDI Mutants Exhibit Defects in Protein Folding Activity—To determine whether the surface-exposed residues that form the hydrophobic patch on CTDI are involved in Sis1 chaperone function, a series of point mutants was constructed (Fig. 2). Then we examined the ability of purified forms of these Sis1 mutants to cooperate with Hsp70 Ssa1 in the refolding of chemically denatured luciferase (Fig. 2, *A* and *B*). When paired with Hsp70 Ssa1, Sis1 K199A, F201H, I203T, and F251S exhibited 70–90% less folding activity than Sis1. In contrast, the protein folding activity of Sis1 V184T and L86Q was similar to that of Sis1. These data demonstrate that Lys-199, Phe-201, Ile-203, and Phe-251 are important for Sis1 to function as a co-chaperone of Hsp70 Ssa1. However, Val-184 and Leu-186 do not appear to be critical for Sis1 to function in the refolding of luciferase.

Sis1 CTDI Mutants Can Stimulate Hsp70 Ssa1 ATPase Activity—For Hsp40 proteins to facilitate luciferase folding they must be able to interact with Hsp70 to stimulate its ATPase activity. To assure that the Sis1 CTDI mutants that exhibited defects in chaperone function retained the ability to interact with Hsp70, their ability to stimulate the ATPase activity of Hsp70 Ssa1 was examined (Fig. 3*A*). All of the Sis1 mutants tested were observed to stimulate the ATPase activity of Hsp70 Ssa1 to the same degree as Sis1. Thus, defects in regulation of Hsp70 ATPase activity do not appear to be responsible for the observed reductions in the protein folding activity of the Sis1 CTDI mutants.

To rule out the possibility that mutation of CTDI caused Sis1 to misfold, thereby hindering its ability to function as a chaperone, we evaluated the folded state of the different Sis1 CTDI mutants. This was accomplished by analyzing the pattern of proteolytic fragments that were liberated by limited digestion of the respective Sis1 mutants by proteinase K. Proteinase K digestion of Sis1 generates proteolytic fragments that correspond to the J-domain and Sis1-(171–352) (Fig. 3*B*). When the protease resistance of purified Sis1 K199A, F201H, and I203T mutants were compared with that of Sis1, we observed no difference in the pattern of fragments formed. In contrast, Sis1 F251S was more sensitive to digestion than the other mutants. The crystal structure of Sis1-(171–352) shows that Phe-251 is located on B5 and forms the base of the depression identified in Sis1 CTDI. Phe-251 is positioned between B1 and B3 and is predicted to promote interactions between these β-strands that stabilize the Sis1 structure (37). Therefore, the observation that Sis1 F251S exhibits increased sensitivity to proteinase K was not surprising. However, this result does hinder our ability

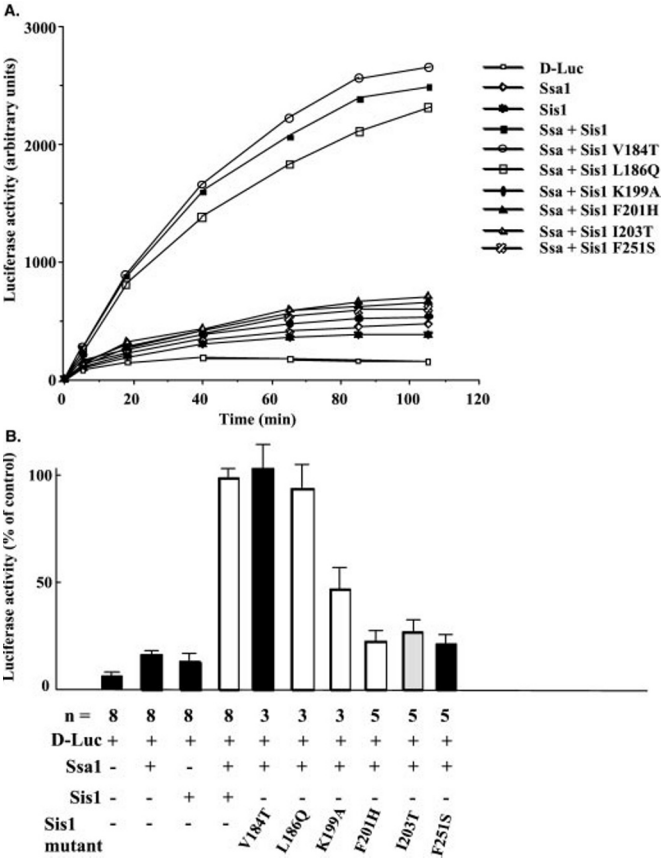

FIG. 2. **Cooperation of Sis1 CTDI mutants with Hsp70 Ssa1 in the refolding of chemically denatured luciferase.** *A*, kinetics of luciferase refolding by purified Hsp70 Ssa1 and Sis1 CTDI mutants. Firefly luciferase (0.04 μM) was denatured with guanidinium HCl and incubated in protein folding buffer (25 mM Hepes, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, and 1 mM ATP) that contained 0.6 μM Ssa1 and 1 μM Sis1 or the indicated Sis1 CTDI mutant. Incubations were at 30 °C, and at the indicated times, aliquots of reactions mixtures were removed and assayed for luciferase activity with a Turner TD20/20 luminometer. Maximal rates of luciferase refolding by Hsp70 Ssa1 and Sis1 occur under the experimental conditions described elsewhere (33). When the Sis1 CTDI mutant proteins were added to reactions at up to 5 μM, we did not observe an increase in rates of luciferase refolding (data not shown). Luciferase activity is expressed in arbitrary units. *B*, quantitation of the relative amounts of luciferase refolded by different Sis1 CTDI mutants. Luciferase activity in reaction mixtures that contained Hsp70 Ssa1 (0.6 μM) and the indicated Sis1 CTDI mutant (1.0 μM) was measured after 60 min of incubation at 30 °C. Values are averages of the indicated number of individual experiments ± S.D. and are expressed as a percentage of the luciferase activity observed when it was refolded by Hsp70 Ssa1 and Sis1.

to make interpretations as to whether Phe-251 is directly involved in Sis1 chaperone function or simply plays a structural role. Nonetheless, defects in the chaperone function observed for Sis1 K199A, F201H, and I203T do not appear to be a result of their defective folding.

Sis1 CTDI Mutants Exhibit Defects in Polypeptide Binding—To test whether the *Sis1* CTDI mutants exhibited defects in polypeptide binding, we utilized an ELISA to analyze their ability to form stable complexes with denatured luciferase (D-Luc; Fig. 4*A*). To validate this ELISA, Sis1 was demonstrated to bind the D-Luc that was immobilized in the wells of microtiter plates in a concentration-dependent manner. Then the inclusion of soluble D-Luc, but not native luciferase, in reactions was shown to competitively block Sis1 binding to immobilized D-Luc. Thus, ELISAs represent a valid tool to monitor complex formation between Sis1 and non-native substrates.

Next, we compared the ability of Sis1 and the CTDI mutants

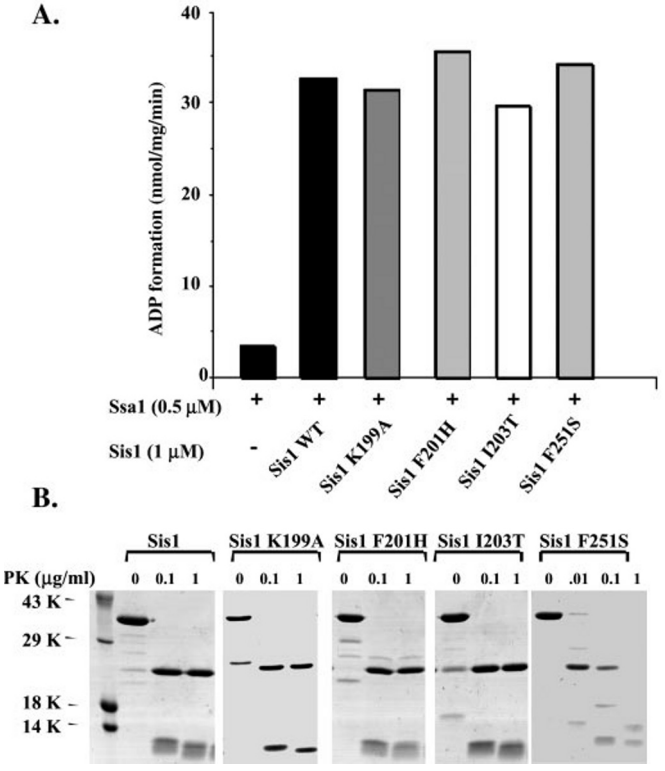

FIG. 3. **Characterization of the Sis1 CTDI mutants folded state and ability to stimulate Hsp70 Ssa ATPase activity.** *A*, stimulation of Ssa1 ATPase activity by CTDI mutants. Purified Hsp70 Ssa1 (0.5 μM) and the indicated form of Sis1 (1 μM) were incubated with [^{32}P]ATP at 30 °C for 10 min. Under these conditions maximal stimulation of Hsp70 Ssa1 ATPase activity is observed. ADP formation was then determined by thin layer chromatography on polyethyleneimine-cellulose plates and scintillation counting. Rates shown represent the average of three independent trials ± S.D. *WT*, wild type. *B*, sensitivity of Sis1 and the Sis1 CTDI mutants to protease digestion. Limited proteolysis of Sis1 (0.3 mg/ml) by proteinase K (*PK*) was performed at 30 °C for 1 h with indicated concentrations of proteinase K. Digested samples were analyzed on 12.5% SDS-polyacrylamide gel electrophoresis and stained with Brilliant Blue R-250. *Arrows* denote the position of Sis1p-(171–352) and two different Sis1 fragments that contain the J-domain.

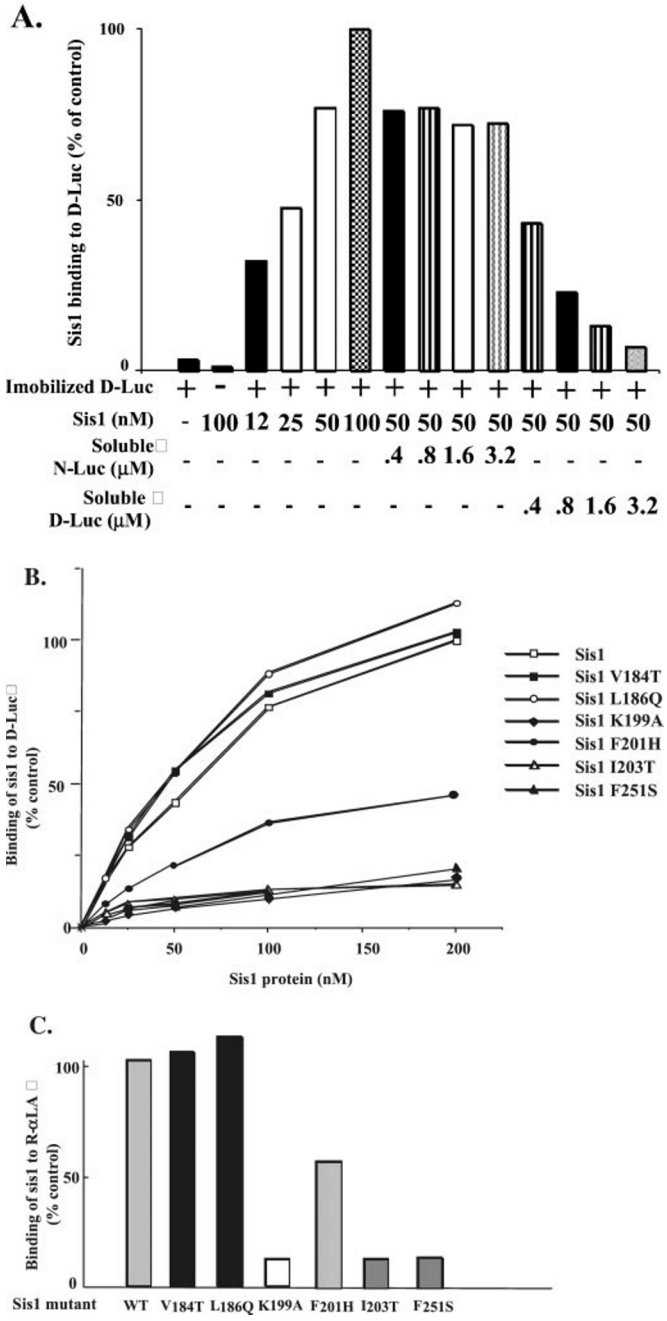

FIG. 4. **Binding of Sis1 mutants to chemically denatured luciferase.** *A*, binding of Sis1 to D-Luc. Firefly luciferase (5 mg/ml) was denatured in 3 M guanidine hydrochloride for 1 h. Then 0.2 μg of D-Luc was immobilized in the wells of microtiter plates (see "Materials and Methods" for details). After luciferase immobilization, Sis1 was added to wells at concentrations that ranged from 12 to 100 nM. Incubations were carried out in a reaction buffer composed of PBST supplemented with 0.2% BSA for 30 min at 25 °C. In competition experiments, the Sis1 concentration was held at 50 nM, and native luciferase or D-Luc was added at the designated concentrations. Retention of Sis1 in wells was detected using a rabbit anti-Sis1 serum and goat anti-rabbit coupled to horseradish peroxidase with color formation being detected at 415 nm. Color formation was linear between 0.1 and 0.6 OD units. *B*, comparison of the binding activity of Sis1 and Sis1 CTDI mutants to D-Luc (100 nM). *C*, quantitation of the binding of Sis1 CTDI mutants (100 nM) to immobilized D-Luc (100 nM). Values are expressed as a percentage of wild-type Sis1 (*WT*) binding.

to bind immobilized D-Luc (Fig. 4, *B* and *C*). Sis1 and the Sis1 V184T and L186Q mutants produced similar binding curves. In contrast, when compared with Sis1, the binding of Sis1 K199A, F201H, I203T, and F251S to D-Luc was reduced from 50 to 75%.

To examine the role of CTDI in the binding a different substrate protein, complex formation between the Sis1 CTDI mutants and a calcium-depleted and reduced form of α-lactalbumin was measured (Fig. 5). R-LA differs from D-Luc in that it has a partially collapsed or molten globule conformation that exposes a number of hydrophobic surfaces and is thought to resemble a late-stage protein-folding intermediate (45, 46). Results from control experiments presented in Fig. 5*A* demonstrate that Sis1 can bind immobilized R-LA in a dose-dependent and conformation-specific manner. Results presented in Fig. 5, *B* and *C*, show that Sis1 V184T and L186Q bind to R-LA with the same efficiency as Sis1. In contrast, the ability of Sis1 K199A, F201H, I203T, and F251S to bind R-LA was reduced from 60 to 85%.

Data obtained from assays that monitor complex formation between Sis1 and two different substrates provide direct evidence that conserved residues lining the hydrophobic depression on CTDI are required for polypeptide binding. These data suggest that the Sis1 CTDI mutants are defective in luciferase folding because they have a reduced capacity to bind denatured luciferase.

Sis1 CTDI Mutants Are Unable to Support Cell Growth—To carry out its essential functions Sis1 requires its J-domain and regions within CTDI (34, 36). Loss of Sis1 CTDI function can be complemented partially by the presence of Ydj1 in the yeast

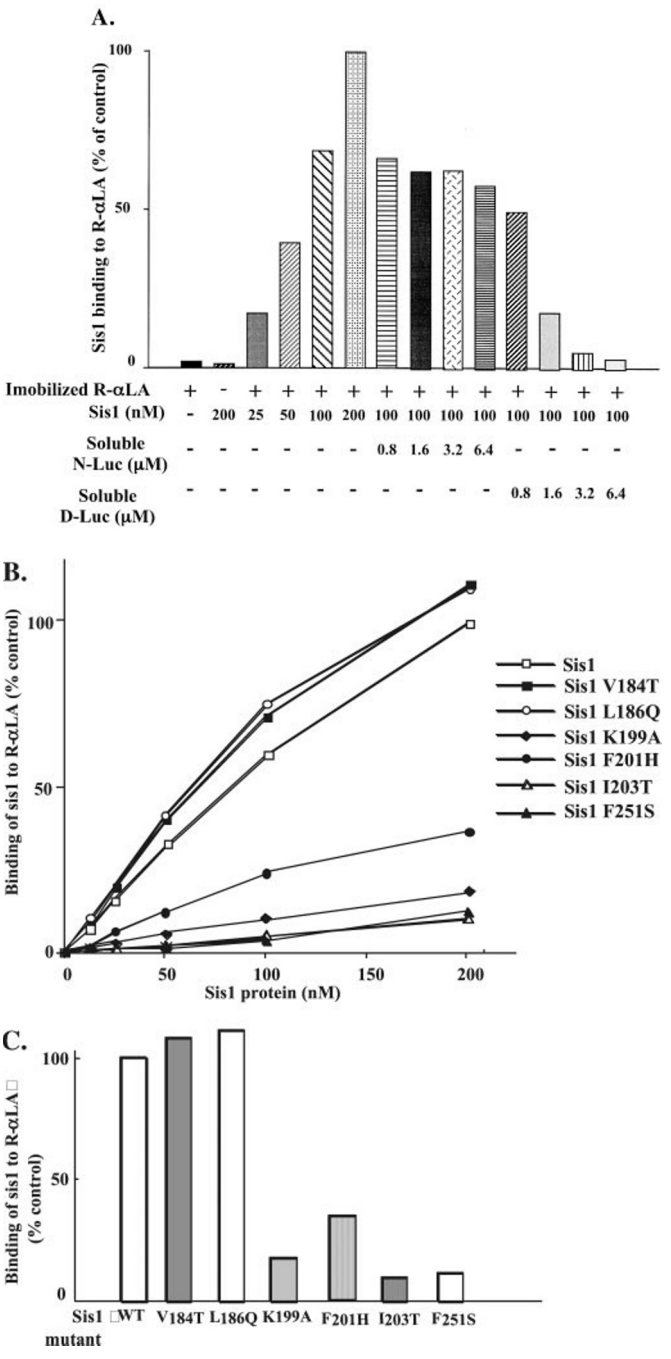

FIG. 5. **Binding of Sis1 CTDI mutants to reduced bovine α-lactalbumin.** *A*, interaction of Sis1 with immobilized R-LA. The fully reduced form of α-lactalbumin (0.4 μg) in a 50-ml volume was immobilized in microplate wells. The binding of Sis1 to R-LA was then monitored via the method described for D-Luc in the legend to Fig. 4, except R-LA was maintained in its reduced molten globule form by the addition of 2 mM DTT to binding reactions. *B*, comparison of the binding of the Sis1 CTDI mutants to R-LA (100 nM). *C*, quantitation of the binding of different forms of Sis1 CTDI mutants (100 nM) to immobilized R-LA (100 nM). Values are expressed as a percentage of wild-type Sis1 (*WT*) binding activity when 100 nM of Sis1 was added.

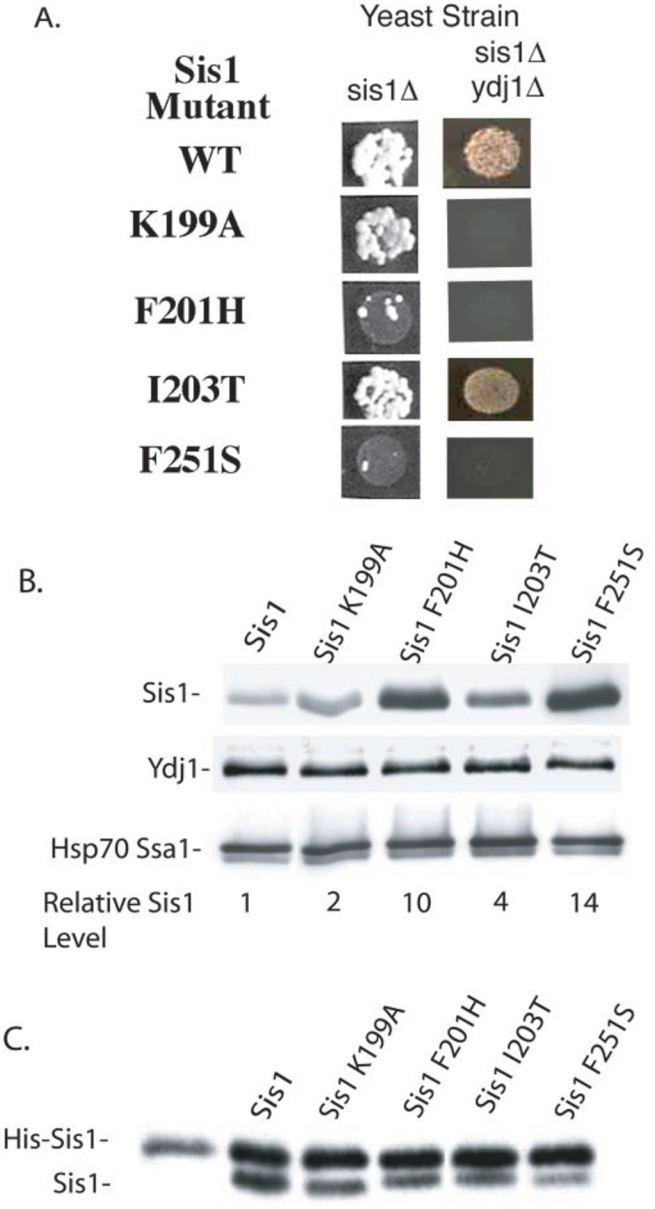

FIG. 6. **Growth phenotypes of *Sis1* CTDI mutants.** *A*, wild-type (*WT*) or mutant versions of *Sis1* were introduced into a *sis1*Δ or *sis1*Δ *ydj1*Δ strain by the plasmid shuffle technique and selected on media that contained 5-fluroorotic acid (44). Strains were grown at 25 °C for 7 days, and then the plates were photographed. *B*, analysis of the expression levels of the different *Sis1* CTDI mutants in the *sis1*Δ strain. The sis1Δ strain harboring *Sis1* or the indicated CTDI mutant was cultured at 25 °C in selective liquid media to an OD of 2.0. Panels from Western blots of cell extracts (5 μg/lane) from trichloroacetic acid-fixed cells were then probed with antibody against Sis1, Ydj1, and Hsp70 Ssa1. The labeling *above* the *lanes* denotes the Sis1 CTDI mutant in which steady state expression level was analyzed. The quantitation *below* in *panel B* represents the ratio of Sis1 CTDI to Sis1 expression. *C*, co-expression of Sis1-His_6 reduces the steady state expression level of Sis1 F201H and Sis1 F251S. The *sis1*Δ strains that harbored the different *Sis1* mutant alleles were cultured as described above, and the steady state level of Sis1 expression was probed by Western blot. The mobility of Sis1-His_6 and Sis1 on 15% SDS-PAGE gels is denoted.

cytosol (34, 36). Thus, we examined the importance of residues that line the hydrophobic depression in Sis1 for its *in vivo* functions by determining the ability of the CTDI mutants to support the growth of *sis1*Δ and *sis1*Δ*ydj1*Δ strains (25, 36). *Sis1 K199A*, *F201H* and *F251S* were not capable of supporting the growth of a *sis1*Δ*ydj1*Δ strain (Fig. 6*A*). Growth defects were also observed when *Sis1 F201H* and *F251S* were asked to support the life of a *sis1*Δ strain, but these strains remained viable (Fig. 6*A*). *Sis1*Δ strains that harbored *Sis1 K199A* and *Sis1 I203T* grew normally (Fig. 6*A*). Thus, it appears the presence of Ydj1 in the cytosol of the *sis1*Δ strains complements the defects in the chaperone function of Sis1 K199T and F201H and allows *sis1*Δ strains that harbor these mutants to grow. However, in the absence of Ydj1, the residues Lys-199, Phe-201, and Phe-251 become essential for Sis1 to maintain cell viability.

Residue Ile-203 in Sis1 was demonstrated to be required for chaperone function *in vitro* but was not observed to be essential *in vivo*. The simplest explanation of this result is that although Ile-203 is important for the binding of some substrates, it is not required for the binding and/or folding of all substrates of Sis1. The results presented support the conclusion that residues in CTDI that are required for polypeptide-binding are also essential in maintaining cell viability.

Expression Levels of Sis1 CTDI Mutants—To assure that the inability of the Sis1 CTDI mutants to support normal cell growth was not caused by decreased expression, the steady state level of the various forms of *Sis1* expressed in the *sis1*Δ strain were compared by Western blot (Fig. 6*B*). Sis1 K199A and I203T were detected at levels near that of Sis1. Interestingly, Sis1 F201H and F251S were detected at levels 10–14 times greater than Sis1. Thus, the Sis1 mutants can be expressed in yeast, and the growth defects observed do not appear to result from reduction in protein levels.

Why are the steady levels of Sis1 F201H and F251S elevated? Sis1 is known to autoregulate its own expression, and the deletion of regions near CTDI causes an induction of *Sis1* expression (47). Thus, defects in the chaperone function of Sis1 F201H and F251S may have caused them to lose their ability to autoregulate their own expression. If this is the case, then the co-expression of Sis1 along with these mutants should return to their steady state levels toward normal. Indeed, this was found to be the case (Fig. 6*C*). Why the F201H and F251S mutants are expressed to higher levels than other Sis1 CDTI mutants such as K199A and I203T, which are also defective in chaperone function, is not clear.

Increased expression of Sis1 F201H and F251S could have dominant negative effects on Hsp70 Ssa1 chaperone action and thereby give rise to the defective growth observed in the *sis1*Δ strains that harbor these mutants. To examine this possibility, Sis1 F201H and F251S were overexpressed from a high copy plasmid in a wild-type and *ydj1*Δ strain and no alteration in the growth rates of either was observed (data not shown). These collective data suggest that mutations in CTDI cause growth defects in yeast because Sis1 mutants cannot perform their essential *in vivo* chaperone functions.

DISCUSSION

The data presented herein support the conclusion that the Type II Hsp40 chaperone protein Sis1 utilizes conserved residues that form a hydrophobic depression on CTDI to bind non-native polypeptides. The function of CTDI in polypeptide binding was demonstrated through the mutational analysis of residues Val-184, Leu-186, Lys-199, Phe-201, Ile-203 and Phe-251, which form a 5-Å deep depression on the surface of this domain. Mutation of these residues compromised the chaperone functions of Sis1 to different degrees; these data are discussed below. Lys-199, Phe-201, and Ile-203 are all located on β-strand 2 in the Sis1-(171–352) structure. The mutation of these residues severely compromised the polypeptide binding and protein folding activity of purified Sis1. Lys-199 is a highly conserved residue in Type II Hsp40s, but it has a charged ϵ-amino group and therefore would not have been predicted to function in polypeptide binding. However, the Sis1-(171–352) structure indicates that the carbon atoms in the side chain of Lys-199 form a portion of the wall of the 5-Å deep hydrophobic depression in CTDI. In addition, the charged ϵ-amino group of Lys-199 is bent away from the interior of the hydrophobic depression and is therefore not predicted to interfere with the binding of hydrophobic amino acids presented by non-native protein substrates of Hsp40s (23). Phe-201 is the least conserved of the residues that line the hydrophobic depression of Sis1, but nonetheless, the aromatic ring in its side chain has a large exposed surface in the wall of the depression in Sis1 CTDI. Ile-203 is a highly conserved residue, and the aliphatic side chain of this residue lies adjacent to the aromatic ring of Phe-201 on the surface of depression Sis1 CTDI. Thus, Lys-199, Phe-201, and Ile-203 all lie adjacent to each other in the Sis1 structure, and these three residues appear to form a hydrophobic surface that is important for the binding of non-native polypeptides. A notable observation was that Lys-199 and Phe-201 are essential for cell viability, but mutation of I203T did not cause any detectable growth defects. A simple explanation for this result is that in the absence of the Ile-203 side chain, the solvent-exposed carbons in Lys-199 and Phe-201 form a hydrophobic surface that is sufficient for Sis1 to bind its essential *in vivo* substrates. However, the chaperone functions of the I203T mutant were clearly compromised because it exhibited severe defects in the binding of two different model substrates.

F251 is located on β-strand 5 and forms the base of the hydrophobic depression on CTDI (37). The Sis1 F251S mutant could not support the growth of the *sis1*Δ*ydj1*Δ strain and exhibited a compromised ability to function as a chaperone. However, purified Sis1 F251S was less resistant to protease digestion than Sis1. Sis1 F251S appeared to fold properly but may be less stable than Sis1 because Phe-251 is likely to form contacts between β-stands 1 and 2 that help stabilize the structure of CTDI. Thus, although Phe-251 is essential for Sis1 chaperone function, whether it simply plays a structural role or actually participates in making contacts with non-native polypeptides is not clear.

Val-184 and Leu-186 are highly conserved residues and thus are predicted to be important for the function of Type II Hsp40s. However, the alteration of Val-184 and Leu-186 did not have a detectable effect on Sis1 function *in vitro* or *in vivo*. In addition to the results reported herein, we have also constructed a V184T,L186Q double mutant, which did not exhibit any detectable functional defects (data not shown). The results obtained with the Val-184 and Leu-186 mutants demonstrate that the aliphatic side chains of Val-184 and Leu-186 can be mutated to more polar side chains and Sis1 still retains its chaperone function. Since the mutational analysis of these residues was not exhaustive, the question of whether or not Val-184 and Leu-186 are important for Sis1 chaperone function requires further examination.

Sis1 and Ydj1 both function with Hsp70 Ssa1 in the yeast cytosol to facilitate different aspects of cellular protein metabolism (24, 25). Sis1 and Ydj1 exhibit differences in their ability to function as chaperones; this observation has been attributed to the fact that regions of these proteins that are implicated as polypeptide-binding domains show limited sequence similarity (27). However, recent genetic studies have shown that the peptide-binding domains of Sis1 and Ydj1 share overlapping essential functions and are likely to bind some of the same *in vivo* substrates (36). In these aforementioned studies the Craig group (36) demonstrated that a fragment of Sis1 that contains the J-domain, G/M region, and CTDI, but not just the J-domain and G/M region, was sufficient to maintain the viability of a *sis1*Δ*ydj1*Δ strain. Based on these data and the prediction from the Sis1-(171–352) structure that CTDI contains a peptide-binding site, it was concluded that function of the substrate binding region of Sis1 was required to maintain the viability of a *sis1*Δ*ydj1*Δ strain. The data we present are in agreement with these studies, and we have extended them by identifying essential residues located in CTDI that enable Sis1 to function in polypeptide binding.

What do the data from the mutational analysis of CTDI on Sis1 tell us about the general nature of the peptide-binding site for Type II Hsp40s? The shape and the size of the depression in

CTDI suggest that this region may only be capable of making contacts with a single residue from a non-native protein. Genetic data from the Lindquist group (4) suggest that, in addition to CTDI, other non-essential regions in Sis1 may also be involved in making contacts with non-native proteins. Sis1chaperone function is required for the maintenance of the [RNQ+] prion (4). Deletion analysis indicates that both the G/F region and the CTD are required for Sis1 to modulate the conformational state of [RNQ+] (4). A direct interaction between the G/F region and [RNQ+] has not been demonstrated, but the data presented suggest that this event does occur. Thus, regions within CTDI may cooperate with other domains within Sis1 to chaperone non-native polypeptides.

The structures of a number of polypeptide-binding proteins have been solved (38). In all of these chaperone proteins, some form of a solvent-exposed hydrophobic region has been found to serve as the binding site for non-native polypeptides. The surfaces of the peptide-binding domains in these chaperones typically contain one or more depressions that influence substrate selectivity (38). Thus, the utilization of a hydrophobic patch on the surface of Sis1 as a component of its polypeptide-binding site fits with the general mechanism for chaperone action previously observed for other protein folding factors. Interestingly, the architecture and valency of different chaperone proteins shows a wide degree of variation. For example, Sis1 is a dimer, and although dimerization is not essential to maintain cell viability, its appears to increase the efficiency of its chaperone action (37). Hsp70 differs from Sis1 in that it functions as a monomer, and access to its peptide-binding groove is regulated by a lid domain (21). Group I chaperonins such as *E. coli* GroEL form a homoheptameric ring that is stacked back-to-back to form a cylinder with two peptide-binding cavities (6, 48). Each monomer within the GroEL ring utilizes a set of conserved hydrophobic residues localized on the apical domain near the mouth of the cavity to bind regions of non-native proteins that are as large as hairpin loops (49). Prefoldin is a hexameric molecular chaperone built from two related classes of subunits and having the shape of a jellyfish. The body of prefoldin is that of a double β-barrel assembly, and it has six arms that have long tentacle-like coil-coil domain structures (22). The distal tips of the coil-coil regions expose hydrophobic surfaces that enable prefoldin to bind to short segments of non-native proteins (22). Thus, although Sis1 is similar to other chaperones in that it utilizes a solvent-exposed hydrophobic surface as a component of its peptide-binding 4site, its homodimeric structure and clamp-like architecture appear to make it structurally unique.

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