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

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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). Hsp40s function as molecular chaperones that bind and target non-native proteins 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 viability and required for Sis1 polypeptide binding activity. Further, the J-domain, Gly and Phe (G/F)-rich region, a zinc finger-like domain, and a conserved carboxyl-terminal domain (CTD). 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 the 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, SIS1-N (5′-ACAGAATCTACATG- GTCAAGGAGACAAACT T-3′) and the 3′-primer, SIS1-C (5′- TGCTTAGGATCCCTATTAAAAATTTCATCTAT AGC-3′). This PCR product was then cloned into the NdeI and BamHI sites present in the polyclinker of the E. coli expression vector pET9a (39) to generate pET9aSis1. To express Sis1 from a plasmid in yeast under the control of its own promoter the primers SIS1-UN (5′-ATGACCATGCATCAATGTCGAGGAGAATTAC T-3′), and the SIS1-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 NdeI and BamHI sites present in the polyclinker of the centromeric yeast expression plasmids pRS314 and pRS315 (40) to generate pRS313Sis1 and pRS315Sis1.

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 StuI and BamHI to generate a DNA fragment that contained bases 148–1056 of Sis1. pET9aSis1, pRS313Sis1, and pRS315Sis1 were then digested with StuI and BamHI, and the mutated and digested Sis1 PCR fragments were then 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 YM medium containing 2% galactose to an A590 of 3. Hsp70 Ssa1 was then purified using ATPagarose, 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 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).

Assay 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 proteasine K (0.1–1.0 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 shown that this method facilitates 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 was utilized (23). A DNAf and its substrates was established (43). This 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 MgCl2, and 5 mM DTT for 1 h at room temperature. Then, 0.5 mg of denatured luciferase-made 0.1 M NaHCO3 (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 incubated with a 1:1000 dilution of peroxidase-conjugated 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% H2O2 and 21 ml of filtered ABTS stock solution (22 mg ABTS in 100 ml of phosphate buffer). 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, Ca2+−depleted; Sigma) at 5 mg/ml was incubated in 10 mM DTT, 0.1 mM Tris (pH 8.7), 0.2 mM KCl, and 1 mM EDTA for 15 min at 37 °C. Then 0.4 μg of reduced LA (R-LA) was added to the wells of microtiter plates in 0.1 mM NaHCO3 (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 state 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 CTD mutants was analyzed by determining whether they could support the growth of a sis1Δ strain (MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 met2-1 lys2-1,100 ydj1-1) on the synthetic complete medium lacking leucine (9). 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 sis1Δ strain was transformed with wild-type or mutant Sis1 that was supplied on a low copy Leu2 plasmid pRS155 (40). The sis1Δ::ydr1Δ strain was transformed with wild-type or mutant Sis1 that was supplied on a low copy Leu2 plasmid pRS155 (40). 
that was supplied on a low copy Trp1 plasmid pRS14 (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 expres-sion 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 A600 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 15% gels the Sis1-His6 protein migrated with a slower mobility than Sis1. This allowed for the visualization of the expression levels of non-tagged Sis1 mutants and Sis1-His6 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

![Figure 1. The domain structure of Sis1.](image-url)

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.
probe the structure of this fragment for solvent-exposed hydrophobic residues (Fig. 1, A and B) and contours (Fig. 1C). 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. 1C). 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. 1D). 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. 3A). 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. 3B). 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 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. 4A). 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
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 H9251-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. 5A 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.
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 \( \text{sis1}^\Delta /H9004 \) and \( \text{sis1}^\Delta \text{ydj1}^\Delta /H9004 \) strains (25, 36).

Sis1 K199A, F201H and F251S were not capable of supporting the growth of a \( \text{sis1}^\Delta /H9004 \) strain (Fig. 6A). Growth defects were also observed when Sis1 F201H and F251S were asked to support the life of a \( \text{sis1}^\Delta \) strain, but these strains remained viable (Fig. 6A). Sis1\( \Delta \) strains that harbored Sis1 K199A and Sis1 F201H and F251S grew normally (Fig. 6A). Thus, it appears the presence of Ydj1 in the cytosol of the \( \text{sis1}^\Delta \) strains complements the defects in the chaperone function of Sis1 K199A and F201H and allows \( \text{sis1}^\Delta \) 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 sis1Δ strain were compared by Western blot (Fig. 6B). 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. 6C). Why the F201H and F251S mutants are expressed to higher levels than other Sis1 CTDI 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 sis1Δ 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 ydj1Δ 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 sis1Δ ydj1Δ 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-strands 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 sis1Δ ydj1Δ 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 domain of Sis1 was required to maintain the viability of a sis1Δ ydj1Δ 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 CTDI are as large as hairpin loops (49). Prefoldin is a hexameric 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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