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Exchangeable Chaperone Modules Contribute to Specification of Type I and Type II Hsp40 Cellular Function

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Hsp40 family members regulate Hsp70s ability to bind nonnative polypeptides and thereby play an essential role in cell physiology. Type I and type II Hsp40s, such as yeast Ydj1 and Sis1, form chaperone pairs with cytosolic Hsp70 Ssa1 that fold proteins with different efficiencies and carry out specific cellular functions. The mechanism by which Ydj1 and Sis1 specify Hsp70 functions is not clear. Ydj1 and Sis1 share a high degree of sequence identity in their amino and carboxyl terminal ends, but each contains a structurally unique and centrally located protein module that is implicated in chaperone function. To test whether the chaperone modules of Ydj1 and Sis1 function in the specification of Hsp70 action, we constructed a set of chimeric Hsp40s in which the chaperone domains of Ydj1 and Sis1 were swapped to form YSY and SYS. Purified SYS and YSY exhibited protein-folding activity and substrate specificity that mimicked that of Ydj1 and Sis1, respectively. In in vivo studies, YSY exhibited a gain of function and, unlike Ydj1, could complement the lethal phenotype of sis1Δ and facilitate maintenance of the prion [RNQ+]. Ydj1 and Sis1 contain exchangeable chaperone modules that assist in specification of Hsp70 function.

INTRODUCTION

Hsp40s direct Hsp70 to facilitate cellular processes that include protein folding, the suppression of amyloid plaque formation, endocytosis, protein translocation across membranes, signal transduction, DNA replication, protein degradation, and prion propagation (Silver and Way, 1993; Cyr *et al*., 1994; Cheetham and Caplan, 1998; Hartl and Hayer-Hartl, 2002). Hsp70 performs its cellular work by using the energy derived from ATP hydrolysis to bind and release protein substrates that display nonnative structure. Hsp40s specify the jobs of Hsp70 by modulating Hsp70s ATP hydrolytic cycle, by acting as chaperone proteins that target substrates to Hsp70 and by influencing Hsp70s subcellular localization.

All Hsp40 family members contain a J-domain that is ~70 amino acids in length, is constructed from four α-helical regions, and is responsible for interactions with Hsp70 (Hill *et al*., 1995; Qian *et al*., 1996). Helix II and a conserved HPD tripeptide located in the J-domain bind to Hsp70 at an acidic groove located in the ATPase domain to stimulate ATP hydrolysis (Greene *et al*., 1998; Suh *et al*., 1998). The conversion of Hsp70-ATP to Hsp70-ADP leads to a conformational change that stabilizes Hsp70: peptide complexes (Langer *et al*., 1992; Bukau and Horwich, 1998).

The Hsp40 family is large and structurally and functionally diverse with members classified into three subtypes (Cheetham and Caplan, 1998). Type I Hsp40s are descendents of *Escherichia coli* DnaJ and are represented by human Hdj-2 and yeast Ydj-1 (Cheetham and Caplan, 1998). Type I Hsp40s contain the J-domain, a G/F rich region, a zinc finger-like domain, and a region termed the conserved carboxyl terminal domain (CTD). Type II Hsp40s, such as human Hdj-1 and yeast Sis1, contain all of the aforementioned domains except that the zinc finger-like region has been replaced by a G/M-rich region and CTDI (Sha *et al*., 2000). Type III Hsp40s contain only the J-domain, and proteins such as yeast Swa2, zuotin, and sec63 have specialized structures that target them to bind specific proteins, nucleic acids, or intracellular membranes (Cyr and Douglas, 1994). Hsp70 family members are often colocalized in the same subcellular compartment with multiple members of the Hsp40 family. Thus, the interaction of a single Hsp70 with different Hsp40s generates specialized Hsp70:Hsp40 pairs that facilitate specific processes at distinct locations within the cell (Caplan *et al*., 1992a,b; Ungermann *et al*., 1994; Liu *et al*., 1998; Meacham *et al*., 1999; Gall *et al*., 2000; Horton *et al*., 2001).

Ydj1 and Sis1 are colocalized in the yeast cytosol with the Hsp70 Ssa1-4 and Hsp70 Ssb1-2 proteins. Ydj1 and Sis1 interact with Hsp70 Ssa proteins, but not with members of the Hsp70 Ssb protein family (Cyr *et al*., 1992; Cyr and Douglas, 1994; Cyr *et al*., 1994; Cyr, 1995). Genetic studies indicate that Ydj1 and Sis1 have specific functional properties that enable them to direct Hsp70 Ssa proteins to facilitate different cellular processes (Caplan and Douglas, 1991; Luke *et al*., 1991; Caplan *et al*., 1992a). For example, the overexpression of Sis1 can complement the slow growth phenotype of ydj1Δ strains, but Ydj1 cannot complement the lethal phenotype of sis1Δ strains (Luke *et al*., 1991). Furthermore, the cellular functions of Ydj1 and Sis1 are different. Ydj1 and its human homolog Hdj2 function on the cytoplasmic face of the endoplasmic reticulum to promote membrane protein folding and protect cells from stress (Caplan *et al*., 1992b; Meacham *et al*., 1999). Whereas Sis1 is found in association

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with translating ribosomes where it facilitates the assembly of translation initiation complexes (Zhong and Arndt, 1993; Horton *et al*., 2001). In addition, Sis1, but not Ydj1, is required of the assembly of the prion [RNQ+] into insoluble fibrils (Sondheimer *et al*., 2001; Lopez *et al*., 2003).

Mechanistic studies with purified Ydj1 and Sis1 demonstrate that both can stimulate Hsp70 Ssa1 ATPase activity to the same degree. But Ydj1 and Sis1 exhibit differences in chaperone function (Lu and Cyr, 1998a,b). The Ydj1/Hsp70 Ssa1 chaperone pair refolds luciferase with severalfold greater efficiency than the Sis1/Hsp70 Ssa1 team (Lu and Cyr, 1998b). Hdj-2 and Hdj-1 exhibit similar differences in chaperone activity (Meacham *et al*., 1999). These collective data demonstrate that type I and type II Hsp40s are not equivalent as chaperones, and we propose that this functional difference plays a role in the specification of Hsp70s cellular action.

The reason why type I and type II Hsp40s exhibit differences in chaperone activity is unknown. Insight into the answer to this question comes from biochemical and structural studies, which suggest that type I and II Hsp40s have evolved to contain structurally distinct polypeptide binding domains. type I Hsp40s seem to use undefined amino acid residues within the zinc finger-like region and an adjacent domain to bind and then deliver nonnative proteins to Hsp70 (Banecki *et al*., 1996; Szabo *et al*., 1996; Lu and Cyr, 1998a). Crystallographic studies suggest that Sis1 and other type II Hsp40s function as divalent homodimers that have a clamp-like architecture and use a shallow groove located on the surface of monomers to bind nonnative proteins (Sha *et al*., 2000; Lee *et al*., 2002). In addition, the G/F regions of Ydj1 and Sis1 lie adjacent to their putative polypeptide binding domains and they seem to influence the functions of these Hsp40s (Yan and Craig, 1999; Johnson and Craig, 2001; Sondheimer *et al*., 2001; Lopez *et al*., 2003).

It is possible that the structural differences exhibited by the chaperone modules of Ydj1 and Sis1 helps confer their ability to specify Hsp70 Ssa1 function in the yeast cytosol. To test this model we constructed a set of chimeric Hsp40s in which the chaperone domains of Ydj1 and Sis1 were swapped to form YSY and SYS. Then we characterized the ability of YSY and SYS to cooperate with Hsp70 Ssa1 to fold proteins, bind substrates, support cell viability, and promote the propagation of the prion [RNQ+]. The experimental results from the aforementioned experiments are presented below and they demonstrate that Ydj1 and Sis1 contain exchangeable chaperone modules that control their protein folding activity and in vivo functions.

MATERIALS AND METHODS

Construction of the Chimeric Hsp40s SYS and YSY

Polymerase chain reaction (PCR) was used to construct chimeric SYS and YSY genes from Ydj1 and Sis1. To construct YSY, the primer (P) P1, GCCACG*CATATG*GTCAAGGAGACAAAAC, and P2, CTTGAAGCTCTTGTGGCTCTTTTCCTGGAT, and the template pET11a-SIS1(Lee *et al*., 2002) were used to generate a DNA fragment that contained bases 1–771 of Sis1. An engineered *Nde*I site in P1 is underlined. The primers P3, ATCCAGGAAAAGAGCCACAAGAGCTTCAAG, and P4, GCAACGGGATCCTTATCATTGAGATGCACATTG, which contains a *Bam*HI site that is underlined, and the template pET9d-Ydj1 (Cyr *et al*., 1992) were then used to generate a DNA fragment that corresponded to bases 768-1218 of YDJ1. The overlapping ends of gel-purified forms of these two fragments were then annealed and used as a template in a PCR reaction primed with P1 and P4 to generate a chimeric gene termed SSY: Sis1 (1–771):Ydj1 (768–1218). SSY was cloned into pGEMT (Promega, Madison, WI) to generate pGEM-SSY.

Next, P5, GCCACGCATATGGTTAAAGAAACTAAG, and P6, ATTGAAAGCATCCTCATTTTGTGCGCCACCAGCACC, and the template pET9d-Ydj1 were used to generate a DNA fragment that contained bases 1–315 of Ydj1 that encoded amino acids 1–105. P7, GGTGCTGGTGGCGCACAAATGAGGATGCTTTCAAT, P4, and the template pGEM-SSY were then used to produce a DNA fragment that corresponded to bases 324–774 of Sis1 that were fused in frame to bases 768-1218 of Ydj1. Next, Sis1 (324–774):Ydj1 (768–1218) and Ydj1 (1–315), which were engineered to have overlapping ends, were annealed and used as a template for a fourth PCR with the Ydj1 primers P5 and P4 to generate the YSY chimeric gene that encodes Ydj1 (1–105):Sis1 (108–257):Ydj1 (256–409).

A strategy similar to the one outlined above was used to produce the SYS chimeric gene. The primers P8, GCCACGCATATGGTTAAAGAAACTAAG, which contains an *Nde*I site that is underlined, P9, TTTAAAAGTTTGGATGTGGTCTCTCAGAAAC, and the template pET9d-Ydj1 were used to generate a Ydj1 fragment that contained bases 1–765 that encoded residues 1–255. Primers P10, GTTTCTGAGAGACCACATCCAAACTTTAAA, and P11, TGCTTAGGATCCCTATTAAAAATTTTCATCTATATAGC, which contains a BamH1 site that is underlined, and the template pET11a-Sis1 were then used to PCR amplify bases 774-1056 of the SIS1 open reading frame that encoded amino acids 258–352. The Ydj1 1–765 and Sis1 774-1056 fragments were engineered to have overlapping ends and thus could be annealed and used as template in a PCR reaction primed with P8 and P11 to generate the YYS chimeric gene that encodes amino acids Ydj1 (1–255):Sis1 (258–352). The YYS fragment was then cloned in pGEMT to generate pGEM-YYS.

P1 and P12, CTTTGTGCGCCACCAGCGCCGCCAAAGAATTGTG, and the template pET11a-Sis1 were then used to amplify a DNA fragment that corresponded to bases 1–363 of Sis1 that encoded amino acid residues 1–121. Primer P12, CACAATTCTTTGGCGGCGCTGGTGGCGCACAAG, and P11 and the template pGEM-YYS were then used to generate Ydj1 303–765:Sis1 774-1056. Next, Sis1 1–363 and Ydj1 303–765:Sis1 774-1056, which have overlapping ends, were annealed and used as a template for a fourth PCR that was primed with P1 and P11 to generate the chimeric SYS gene that contained amino acids Sis1 (1–121):Ydj1 (101–255):Sis1 (258–352).

Once constructed, YSY and SYS were digested with *Nde*I and *Bam*HI, and the DNA fragments generated were ligated into the pET-11a *E. coli* expression plasmid. For construction of a SYS yeast expression plasmid, pGEM-SYS was cut at a *Hin*dIII site that was inside the first S portion of SYS and at the engineered 3′ *Bam*HI. This SYS fragment was then used to replace a similar fragment cut from the Sis1 gene harbored in pRS315-Sis1 under the control of it own promoter (Lee *et al*., 2002). To generate pRS315-YSY, pGEM-YSY was digested at an internal *Pst*I site in the first Y portion and at the engineered 3′ *Bam*HI. This fragment was then ligated into similarly digested pRS315-Ydj1 that was under control of the Ydj1 promoter. The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to mutate YSY and SYS to generate YSY F201H and SYS C201S, respectively. A similar approach used to generate Sis1 N108I D110G and SYS N108I D110G.

Protein Purification

Ydj1, Sis1, and the indicated chimeric Hsp40 or Hsp40 mutant were overexpressed in *E. coli* strain BL21 (DE3) by induction with 0.5 mM isopropyl β-D-thiogalactoside followed by growth for 3 h at 30°C. Hsp40s were then purified by ion exchange and hydroxyapatite chromatography (Lu and Cyr, 1998a,b). Hsp70 Ssa1 was purified by ATP-agarose chromatography from yeast strain MW141 (Cyr *et al*., 1992). Purified proteins were dialyzed against 20 mM HEPES, 150 mM NaCl, 10 mM dithiothreitol (DTT), concentrated to 1–2 mg/ml, and then snap frozen in liquid N_2 or stored on ice before use.

Assay of Hsp70 Ssa1- and Hsp40-dependent Refolding of Denatured Luciferase

Refolding of denatured luciferase by Ssa1 and Hsp40 chaperone pairs was performed as described previously (Lee *et al*., 2002). Briefly, firefly luciferase (13.3 mg/ml; Promega) was diluted 42-fold into 25 mM HEPES, pH 7.4, 50 M KCl, 5 mM $MgCl_2$, 6 M guanidinium-HCl, 5 mM DTT and denatured by incubation at 25°C for 1 h. Aliquots (1 μl) were removed and added to 124 μl of refolding buffer that was composed of 25 mM HEPES, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 1 mM ATP and contained 0.5 μM Ssa1 and Hsp40 chaperone proteins at 1.0 μM. Reaction mixtures were incubated at 30°C, and at the indicated times, 1-μl aliquots were removed and mixed with 60 μl of luciferase assay buffer (Promega). Luciferase activity was then determined with a Turner TD 20/20 luminometer.

Measurement of Hsp70 Ssa1 ATPase Assay

Ssa1 ATPase activity was measured according to a previously established protocol (Cyr *et al*., 1992). Ssa1 (0.5 μM) and Hsp40 proteins (1.0 μM) were added to reaction cocktails that contained 20 mM HEPES, pH 7.4, 80 mM KCl, 10 mM DTT, 1 mM $MgCl_2$, 50 μM[α-^{32}P]ATP (2.0×10^5 cpm/pmol) and incubated for 10 min at 30°C. Thin-layer chromatography on PEI cellulose plates and scintillation counting was then used to determine ADP formation.

Measurement of Polypeptide Binding by Hsp40

A previously established enzyme-linked immunosorbent assay (ELISA) was used to measure complex formation between Hsp40 proteins and denatured luciferase (Lee *et al*., 2002). Hsp40s were diluted into 50 mM phosphate, 150 mM NaCl, pH 7.4 (phosphate-buffered saline, PBS). Then, 100-μl aliquots of

25–200 nM solutions of the indicated Hsp40 were added to the wells of microtiter plates. Hsp40s were allowed to adhere to the walls of wells during a 1-h incubation at 25°C. Wells were then washed to remove unbound Hsp40 with 50 mM phosphate, pH 7.4, 150 mM NaCl, 0.02% Triton X-100 (phosphate-buffered saline/Tween 20, PBST). Wells were then blocked via a 1-h incubation with 200 μl of 0.5% bovine serum albumin (BSA) in PBS. Chemically denatured luciferase (0.4 μg) in PBS that contained 0.02% Triton X-100 and 0.2% BSA was then added to each well. After 1-h incubation at 25°C, the wells were washed three times with PBST. Luciferase retained in the wells was then detected via an ELISA that used rabbit α-luciferase (Cortex Biochem, San Leandro, CA). α-Luciferase was diluted 1:5000 in PBST that contained 0.2% BSA and was incubated in the wells for 1 h at 25°C. After three washes with PBST, goat α-rabbit serum coupled with horseradish peroxidase was used to detect the α-luciferase that was retained in the wells. Color formation was measured at 405 nm with a microplate reader (Bio-Rad, Hercules, CA).

Biopanning of a 7meric Coliphage Peptide Display Library with Ydj1 and Sis1

A coliphage display library that expressed seven amino acid residue peptides at the amino terminus of the M13 coat protein PIII, Ph.D.-7 Peptide Library, was purchased from New England Biolabs (Beverly, MA). The library was screened for peptides preferentially bound by Ydj1 and Sis1 according to the instructions of the manufacturer. Solutions of respective Hsp40 chaperones (100 μg/ml) that contained 0.1 M $NaHCO_3$ (pH 8.6) were immobilized on a polystyrene petri dish (15 × 60 mm) by overnight incubation at 4°C. The Hsp40-coated dish was then washed and blocked with a solution that contained 5 mg/ml BSA and 0.1 M $NaHCO_3$ for 1 h at 4°C. The coated and blocked dish was then washed with Tris-buffered saline/Tween 20 (TBST) (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) six times. The Hsp40-coated dish was then incubated for 30 min with 1 ml of the 7meric library that was diluted with TBST to a titer of 4×10^{10} coliphage/ml. The plate was then washed with TBST, and the tightly bound coliphage was eluted by addition of a TBS solution that contained Ydj1 or Sis1 at 100 μg/ml. Coliphage present in the eluate was then amplified by infection of a 20-ml culture of early log phase *E. coli* ER2537 that was incubated for 4.5 h at 37°C with vigorous shaking. The titer of the amplified coliphage was determined, and the second and third rounds of biopanning were performed. Later rounds of biopanning were carried out in a manner similar to the first, except as per the instructions from New England Biolabs, the stringency of the washes was enhanced by increasing the Tween 20 concentration in TBST to 0.2% in the second round and 0.5% in the third. After the third round of panning, the coliphage eluate was plated and the coliphage present in isolated plaques was purified and the DNA was sequenced on both the sense and anti-sense strand. Via this method, we determined the nature of the peptides that can be recognized by Ydj1 and Sis1.

Complementation of ΔSis1 Strain by SYS and YSY

SL001 (MATa *ade2-1 his3-11, 15 leu2-3, 112, ura3-1, trp1-1, ssd1-d2, cab1-100, sis1::HIS*; pRS316-SIS1) was transformed with the indicated pRS315 plasmids (Lee *et al.*, 2002). pRS316 contains the URA marker and pRS315 contains the Leu marker. To exchange the pRS316-SIS1 plasmid for the respective pRS315 plasmids, transformants were grown on synthetic dextrose (SD) plates that were supplemented with adenine (20 μg/ml) and tryptophane (20 μg/ml). To counterselect the pRS316 plasmid, transformants were then cultured in liquid media and spotted on SD plates that were supplemented with adenine (20 μg/ml), tryptophan (20 μg/ml), and uracil (20 μg/ml), and 0.1% 5-fluoroorotic-acid (5-FOA). Colonies that grow on 5-FOA plates are those that have lost pRS316-SIS1 but retain a pRS315 that harbors the indicated Hsp40 gene.

Western Blot Analysis of SYS and YSY Expression

The indicated yeast strains were cultured in YPD liquid media at 30°C for 2 d. Cells were isolated by centrifugation at 5000 rpm for 5 min and then disrupted by agitation with 0.5-mm glass beads. Cell lysis was achieved by six repeated periods of vortexing for 30 s that were interrupted by incubation of samples for 1 min on ice. Debris was cleared from lysates by centrifugation at 10,000 rpm for 10 min. Proteins in cell extracts were separated on 12.5% SDS-PAGE gels and transferred to a nitrocellulose membrane. The indicated Hsp40s were then detected with rabbit α-Sis1 that was used at a 1:10,000 dilution in PBS supplemented with 0.02% Triton X-100 and 5% nonfat dry milk. Goat α-rabbit horseradish peroxidase conjugate was used at a 1:10,000 dilution to detect the α-Sis1 that decorated the membrane. Blots were developed with the enhanced chemiluminescent reagent (Amersham Biosciences, Piscataway, NJ).

Fluorescence Microscopy

Strain SL001 (sis1Δ) harboring Sis1 and the indicated chimeric Hsp40 genes in pRS315 were transformed with pRNQ1-GFP (Sondheimer *et al.*, 2001). Strains were cultured overnight at 30°C and then Rnq1-GFP expression was induced from the CUPI promoter in pRNQ1-GFP by addition 50 μM $CuSO_4$. After 4 h of induction, aliquots of cells placed on glass slides and viewed with a Nikon fluorescence microscope. Images obtained were modified for publication with Adobe PhotoShop 6.0 software.

Analysis of Rnq1 Aggregation

Analysis of Rnq1 aggregation was carried via a modified version of a previously described assay (Sondheimer *et al.*, 2001). Yeast cells were lysed with glass beads in buffer composed of 75 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.5 mM EDTA, 2.5% glycerol, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride. Cell extracts were centrifuged at 10,000 rpm for 5 min, and supernatants were subjected to ultracentrifugation at 280,000 × *g* for 1 h in a TLA-100 rotor in an Optima TL ultracentrifuge (both form Beckman Coulter, Fullerton, CA). Samples were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were then decorated with Rnq1 antibody (from S. Lindquist, Whitehead Institute for Biomedical Research, Cambridge, MA) at 1:5000 dilution and developed with the enhanced chemiluminescence kit from Amersham Biosciences.

Coimmunoprecipitation of Rnq1 with Sis1

Strain SL001 (sis1Δ) harboring Sis1 and the indicated chimeric Hsp40 genes was grown at 30°C to a density of 3 OD_{600}/ml and then lysed with glass beads in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail from Roche Diagnostics, Indianapolis, IN). Lysis was conducted by placing samples on a vortex for 30 s, cooling them on ice 30 s, and then repeating this process six times. Cell extracts were cleared of debris by centrifugation at 10,000 rpm for 5 min. The cleared supernatant was removed and supplemented with 1 μl of preimmune or Sis1 polyclonal antisera. Samples were incubated at 4°C on a rotator for 1 h and then supplemented with protein G agarose beads. After an additional 1-h incubation, the beads were pelleted and washed with lysis buffer four times. The washed beads were then resuspended in 30 μl of 2× SDS loading buffer and heated at 90°C for 15 min. The contents of samples were then resolved on a 10% SDS-PAGE gel and probed for the presence of Sis1 and Rnq1 by Western blot.

RESULTS

Construction of Chimeric Ydj1 and Sis1 Proteins

To examine the role that the conserved protein modules located in the middle of Ydj1 and Sis1 play in regulating Hsp70 Ssa1 function, we constructed a set of chimeric proteins in which the chaperone domains of these Hsp40s were exchanged to generate YSY and SYS (Figure 1). These domains are termed chaperone domains because there is evidence that they function to enable Hsp40 to function as molecular chaperones to assist Hsp70 in protein folding. The domain boundaries used for the construction of these chimeras were determined with the aid of the Sis1 (171–352) crystal structure (Sha *et al.*, 2000) and sequence alignments (Caplan and Douglas, 1991). The most highly conserved regions between Sis1 and Ydj1 are located in the N and C termini (Caplan and Douglas, 1991; Luke *et al.*, 1991). Sequence alignments show that the N termini of Ydj1 and Sis1 diverge at the end of the G/F region around residue 107 of Sis1. The Sis1 (171–352) crystal structure demonstrated that the C terminus of Sis1 can be divided into CTDI and CTDII and a dimerization domain (Sha *et al.*, 2000). Regions within CTDII and the dimerization domain of Sis1, but not CTDI, are conserved in Ydj1 and DnaJ. The boundary between Sis1 CTDI and CTDII contains an H-X-X-FKRDGDDL motif that corresponds to residues 257–267, and this motif is also present in Ydj1 (Caplan and Douglas, 1991). Therefore, it was reasoned that H-X-X-FKRDGDDL might represented a splice site at which the chaperone domains of type I and type II Hsp40s were fused to CTDII. Based on the above-mentioned considerations, the Sis1 chaperone module was postulated to lie between amino acid residues 108–257, which contains the G/M-rich region and CTDI.

In the case of Ydj1, we knew from previous work that the fragment Ydj1 (179–384), which is missing the first zinc binding motif and contains CTDII, retains chaperone function (Lu and Cyr, 1998a). Based on the data obtained with Ydj1 (179–384) and the sequence identity between CTDII of

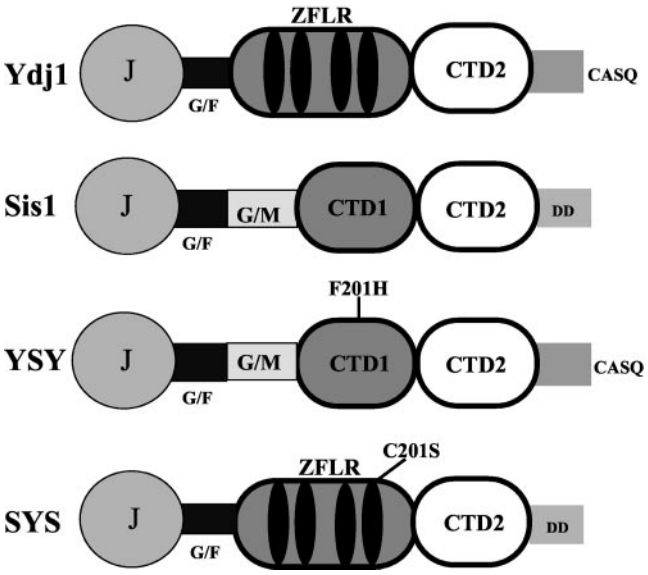

Figure 1. Domain structure of SYS and YSY chimeric proteins. YSY contains the following amino acids: Ydj1 (1–105):Sis1 (108–257):Ydj1 (256–409). The SYS contains amino acids Sis1 (1–121):Ydj1 (101–255):Sis1(258–352). The zinc finger like region (ZFLR) and adjacent C-terminal residues in Ydj1 that are proposed to function in polypeptide binding are located between amino acids 101 and 255. The locations where mutations in the polypeptide binding sites of YSY F201H and SYS C201S are located are denoted. Please see MATERIALS AND METHODS for details about the construction of SYS and YSY.

Sis1 and Ydj1, we reasoned that the substrate-binding site of Ydj1 was located somewhere between residues 179–255. However, it is likely that the proper folding of Ydj1 requires an intact ZFLR. Therefore, we chose to designate a fragment that contained both zinc binding regions of the Ydj1 ZFLR that terminated at the border between the ZFLR and CTDII, residues 101–255, as the Ydj1 chaperone module. Based on this rationale, we used PCR to construct YSY and SYS in which the aforementioned chaperone modules of Ydj1 and Sis1 were swapped.

To generate a set of control chimeric Hsp40s, the chaperone module mutant chimeras YSY F201H and SYS C201S were constructed (Figure 1). F201 in the Sis1 chaperone domain was chosen for mutation in YSY because this residue was previously demonstrated to be located in a surface exposed hydrophobic patch on CTDI that functions in polypeptide binding (Sha *et al*., 2000, Lee *et al*., 2002 #609). Sis1 F201H folds properly and is required for Sis1 to efficiently bind and refold denatured luciferase (Lee *et al*., 2002). The C residue at position C201 in the ZFLR of SYS was chosen for mutation to S because we have observed that Ydj1 C201S is properly folded but exhibits defects in luciferase folding activity and is therefore required for Ydj1 chaperone function (Fan and Cyr, unpublished observation). Please see MATERIALS AND METHODS for details about the construction of YSY and SYS.

The Chaperone Modules of Ydj1 and Sis1 Specify the Luciferase Folding Activity Hsp70 Ssa1

To determine whether the chaperone modules located within Ydj1 and Sis1 influence the protein folding activity of Hsp70 Ssa1, we compared the luciferase folding activity of YSY and SYS to that of Ydj1 and Sis1 (Figure 2A). As previously reported (Lu and Cyr, 1998b), Ydj1 stimulated the luciferase refolding activity of Hsp70 Ssa1 ~10-fold. Compared with the Ydj1:Hsp70 Ssa1 team, Sis1:Hsp70 Ssa1 refolded around 2.5-fold less luciferase (Figure 2A). SYS exhibited a gain of function and cooperated with Hsp70 Ssa1 to refold denatured luciferase with the same efficiency as Ydj1. In contrast, YSY exhibited a loss of folding activity and functioned with Hsp70 Ssa1 to refold denatured luciferase with the same efficiency as Sis1. Compared with Ydj1 and SYS, SYS C201S exhibited an 80% reduction in luciferase folding activity. The defect in luciferase folding activity exhibited by SYS C201S was similar to that exhibited by Ydj1 C201S (Fan and Cyr, unpublished data). Compared with Sis1 and YSY, YSY F201H exhibited an 80% reduction in protein folding activity. The defect luciferase folding activity exhibited by YSY F201H was as severe as that exhibited by Sis1 F201H (Lee *et al*., 2002).

To determine why YSY and SYS refold luciferase with different efficiencies, the ability of these chimeric Hsp40s to interact with Hsp70 Ssa1 and stimulate its ATPase activity was compared with that of Ydj1 and Sis1 (Figure 2B). The chimeric Hsp40s under study all stimulated the ATPase activity of Ssa1 to the same degree. Thus, differences in ability to regulate Hsp70 Ssa1 ATPase activity cannot account for the differences in protein folding activity exhibited by YSY and SYS.

Next, the ability of Ydj1 and Sis1 to bind denatured luciferase was compared with that of YSY and SYS (Figure 2C). Ydj1 bound around 20% more denatured luciferase than Sis1. Polypeptide binding by YSY and SYS mimicked that of Ydj1 and Sis1, respectively. YSY F201H and SYS C201S both exhibited a statistically significant 50% reduction in complex formation with nonnative luciferase. The defects in substrate binding exhibited by YSY F201H and SYS C201S were as severe as those observed in Sis1 F201H (Lee *et al*., 2002) and Ydj1 C201S (Fan and Cyr, unpublished observation). The luciferase binding data presented demonstrate that Ydj1 and SYS bind similar quantities of chemically unfolded luciferase as Sis1 and YSY. Thus, difference in simple ability of YSY and SYS denatured luciferase cannot explain to the differences in protein folding function they displayed.

Nonetheless, the data presented in Figure 2 demonstrate that YSY and SYS are functional Hsp40s whose protein folding activity mimics that of Sis1 and Ydj1, respectively. Thus, the chaperone modules of type I and type II Hsp40s control the luciferase folding activity of Ydj1 and Sis1, but the mechanism by which they act is not clear.

Determination of the Substrate Specificity of Ydj1 and Sis1

A plausible reason to explain why the chaperone modules of Ydj1 and Sis1 enable Hsp70 Ssa1 to refold luciferase with different efficiencies is that they exhibit a difference in substrate specificity. Such a mechanism might enable Ydj1 to preferentially bind folding intermediates of luciferase, which expose a more limited set of binding motifs than unfolded forms, with higher affinity than Sis1. Such a mechanism has the potential to allow Ydj1:Hsp70 Ssa1 to refold luciferase with greater efficiency than Sis1:Hsp70 Ssa1.

To obtain experimental support for this aforementioned model, the substrate specificity of Ydj1 and Sis1 was investigated via screening a 7meric coliphage peptide display library that contained 1.9×10^9 different peptides (Figure 3). The coliphage used in these experiments carries a random seven-amino acid extension that is linked by a $-G_3$-S-sequence to the N terminus of the pIII surface protein of coliphage M13. A 7meric peptide display library was chosen

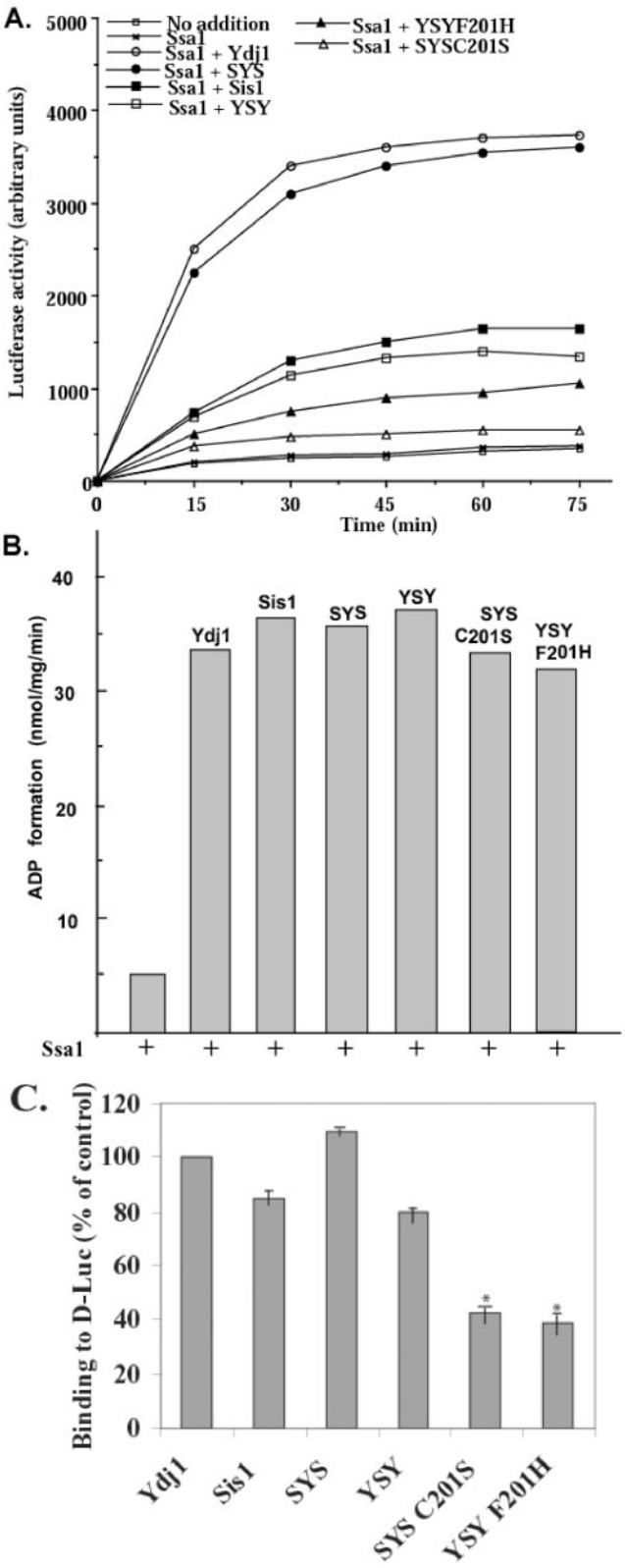

Figure 2. Refolding of denatured luciferase by SYS and YSY. (A) Kinetics of luciferase refolding by Ssa1 and Hsp40 proteins. Guanidinium HCl denatured luciferase was incubated with Ssa1 (0.5 μM) and different Hsp40 proteins (1 μM) at 30°C. At the indicated times, aliquots of the refolding reaction were removed and luciferase activity was determined with a Turner TD-20/20 luminometer. Luciferase activity is expressed in arbitrary units as percentage of total activity observed when it was refolded by Ydj1 and Ssa1. (B) Stimulation of Ssa1 ATPase activity by SYS and YSY. Ssa1 (0.5 μM) and Hsp40 (1.0 μM) were mixed in reaction buffer (20 mM HEPES, pH 7.4, 80 mM KCl, 10 mM DTT, 1 mM $MgCl_2$, 50 μM [α-^{32}P]ATP; ICN Biomedicals, Costa Mesa, CA) at 30°C for 10 min. Then, 2-μl aliquots of reaction cocktails were loaded on PEI cellulose plates that were developed with a LiCl mobile phase. ADP formation was then measured by liquid scintillation counting. The results shown represent the average of two assays. (C) Complex formation between SYS and YSY and denatured luciferase. Aliquots (100 μl) of Hsp40 at 100 nM in PBS were immobilized in the wells of 96-well microtiter plates. Then, chemically denatured luciferase (0.4 μg) was added to wells. ELISA was then used to detect the luciferase that was retained in washed wells. Values are expressed as a percentage of the level of complex formation between immobilized Ydj1 and denatured luciferase. Results are average of three trials ± SD. The asterisk (*) denotes values that were significantly different from the control with a $P > 0.05$.

for these studies because the crystal structure of the Sis1 polypeptide binding domain predicts Sis1 to make contacts with the side chains of one or two solvent exposed residues in nonnative proteins (Sha *et al.*, 2000; Lee *et al.*, 2002). In addition, the Ydj1 homolog DnaJ has been observed to bind peptides with a hydrophobic core of 4–5 hydrophobics that are flanked by charged or polar residues (Rudiger *et al.*, 2001). Therefore, a 7meric peptide library was surmised sufficient to mimic the binding motifs in nonnative proteins that are recognized by Hsp40s.

Ydj1 and Sis1 each selected phage displayed peptides that were enriched in hydrophobic amino acids, but clear differences in the amino acid composition of the peptides selected by these Hsp40s were observed (Figure 3A). Ydj1 selected 17 peptides, 10 of which were distinct, that contained an H at position 2 and had a hydrophobic core of three to four amino acids. Of these peptides G-H-I-I-Y-L-S and S-H-T-I-Y-L-S were selected three and five times, respectively. The hydrophobic core found in these phage displayed peptides is similar to that found in the Hsp70 peptide binding motif and is also present in regions of luciferase that are recognized by DnaJ (Rudiger *et al.*, 1997, 2001). All the other Ydj1-selected peptides were enriched in hydrophobic residues but did not contain an extended hydrophobic core. When the enrichment of amino acids in the Ydj1-selected peptides was compared with the residues present in peptides displayed on 70 different unselected phage from the library W, H, Y, I, F, and S were clearly enriched. In contrast, G, E, A, K, P, M, Q and V were excluded (Figure 3, B and C).

Peptides selected by Sis1 were enriched in hydrophobic amino acids, but the majority did not contain more than two sequential hydrophobic residues. A notable feature of Sis1 peptides was that 11 of 34 had a P at position 6 (Figure 3A). Another set of six peptides had a P at position 2. Most Sis1-selected peptides were enriched in aromatic hydrophobic residues and K. When the ratio of the amino acid occurrence in the selected peptides was compared with 70 different nonselected peptides, W and K were enriched two- to threefold, whereas I, Y, and V were modestly enriched (Figure 3, B and C). In contrast, D, E and G seemed to be excluded from the selected peptides.

If Ydj1 and Sis1 are capable of recognizing different aspects of protein structure, then they should exhibit selectivity toward the substrates they selected from the coliphage peptide display library. To test this supposition, we compared the relative binding of Ydj1 and Sis1 for a subset of the coliphage-displayed peptides that were selected by each other (Figure 4). To accomplish this objective, first we exam-

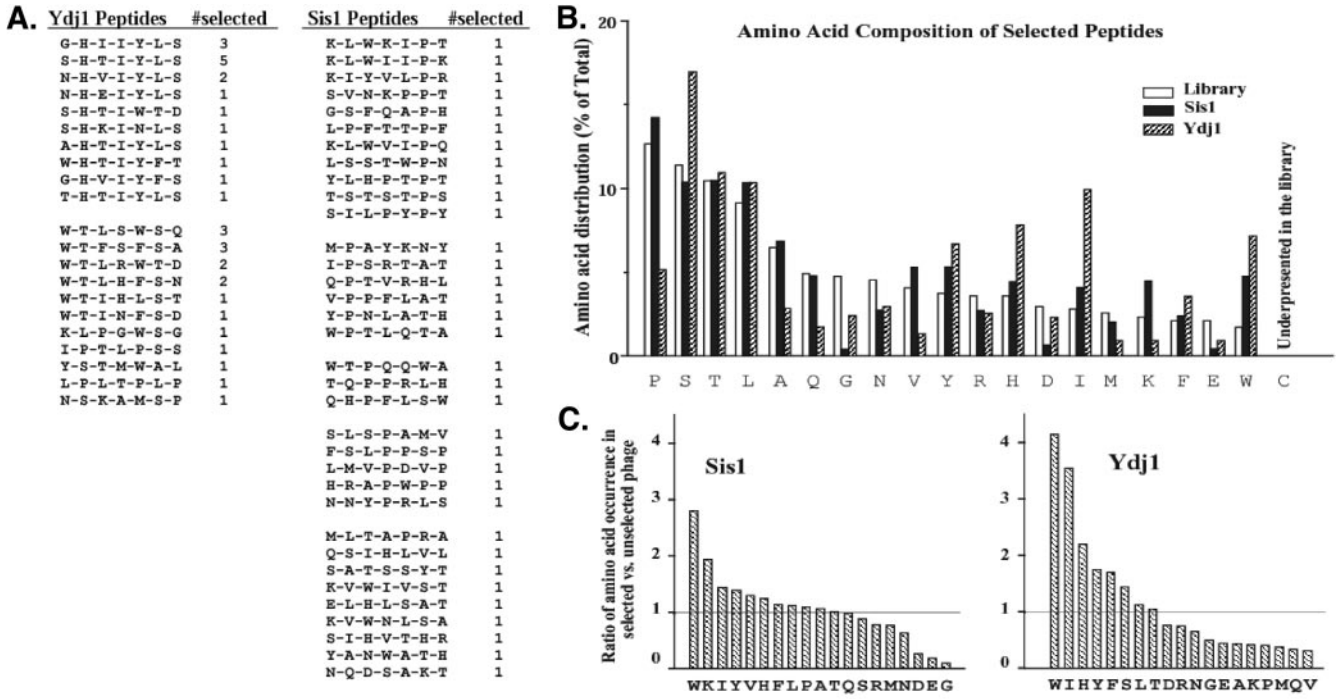

Figure 3. Biopanning of a coliphage 7meric peptide display library with purified Sis1 and Ydj1. (A) Amino acid sequence of the peptides selected by Ydj1 and Sis1. The selection of peptides by Ydj1 and Sis1 was as described in MATERIALS AND METHODS. (B) Distribution of individual amino acid residues in the Hsp40-selected peptides. (C) Ratio of amino acid residues in respective populations of Ydj1- and Sis1-selected peptides relative to that of 70 different unselected peptides from the 7meric peptide display library.

ined the ability of Ydj1 and Sis1 to form complexes with a subset of the coliphage-displayed peptides that they individually selected. Ydj1 bound the peptides GHVIYFS, GHIIYLS, and WTFSFSA with the highest affinity (Figure 4A). SHTIYLS whose central core of four hydrophobic residues differs from GHVIYFS by the substitution of T for V at position 3 was bound with moderate affinity. Sis1 exhibited high relative binding to the peptides that contained multiple P residues, FSLPPSP, and HRAPWPP (Figure 4B). WPTLQTA and YPNLATH, which had a P at position 2, were bound with moderate affinity. MLTAPRA had a P at position 3 was bound by Sis1 with lowest affinity.

Next, the ability of Ydj1 and Sis1 to bind their respective high-affinity substrates was compared (Figure 4, A and B). Ydj1 exhibited very low affinity for the P-enriched peptides preferred by Sis1, FSLPPSP, and HRAPWPP (Figure 4A). Likewise, Sis1 bound the Ydj1 substrate GHVIYFS with very low relative binding affinity. However, Sis1 could bind the Ydj1 substrate WTFSFSA, which lacks a hydrophobic core, and resembles some of the Sis1 selected peptides, with an affinity that was similar to that observed for FSLPPSP (Figure 4B).

To demonstrate that complex formation between Ydj1 and Sis1 and the coliphage displayed peptides mimicked the binding of nonnative polypeptides to Hsp40s, the ability of Ydj1 C201S, Sis1 F201H, and Sis1 I203T to bind phage displayed peptides was examined (Figure 4, C and D). Compared with Ydj1, Ydj1 C201S bound ~90% less coliphage GHVIYFS. Compared with Sis1, Sis1 F201H and Sis1 I203T exhibited a 65–80% decrease in complex formation with coliphage FSLPPSP. These data demonstrate that mutations in the polypeptide binding domains of Hsp40, which cause defects in substrate binding, hinder the ability of Ydj1 and Sis1 to bind phage-displayed peptides. Thus, Ydj1 and Sis1 seem to use their polypeptide binding grooves to form complexes the different coliphage displayed peptides.

In competition experiments, denatured luciferase and denatured lactalbumin were observed to competitively block complex formation between Sis1 and coliphage FSLPPSP and Ydj1 with coli GHVIYFS with half-maximal inhibition observed at 1–2 μM concentrations of nonnative protein (our unpublished data). Synthetic peptides that corresponded to FSLPPSP and GHVIYFS were also observed to inhibit the formation of Hsp40:substrate complexes. However, half-maximal inhibition of complex formation was only observed at peptide concentrations of 100–200 μM (our unpublished data). Thus, although it seems that coliphage-displayed peptides are specifically recognized by the Hsp40 polypeptide binding domains, synthetic peptides that mimic the phage displayed peptides function poorly as inhibitors of Ydj1 and Sis1 chaperone function. M13 displays five copies of individual peptides they display on their surface. Because Ydj1 and Sis1 seem to function as dimers, stable complex formation between them and substrates may require occupancy of the polypeptide binding site located on each monomer. However, 7meric synthetic peptides are not long enough to occupy more than one Hsp40 binding site at a time. This factor may contribute to inability of synthetic phage display peptide to effectively compete for substrate binding to Sis1 and Ydj1.

Comparison of the sequences and amino acid composition of the pools of selected peptides in combination with the binding affinity data that we obtained reveals a number of similarities and differences in the substrate specificity of Ydj1 and Sis1. The coliphage-displayed peptides selected by each of these Hsp40s were enriched in aromatic and hydrophobic residues. W is one of the least abundant residues in the library, but it was enriched in the peptides selected by

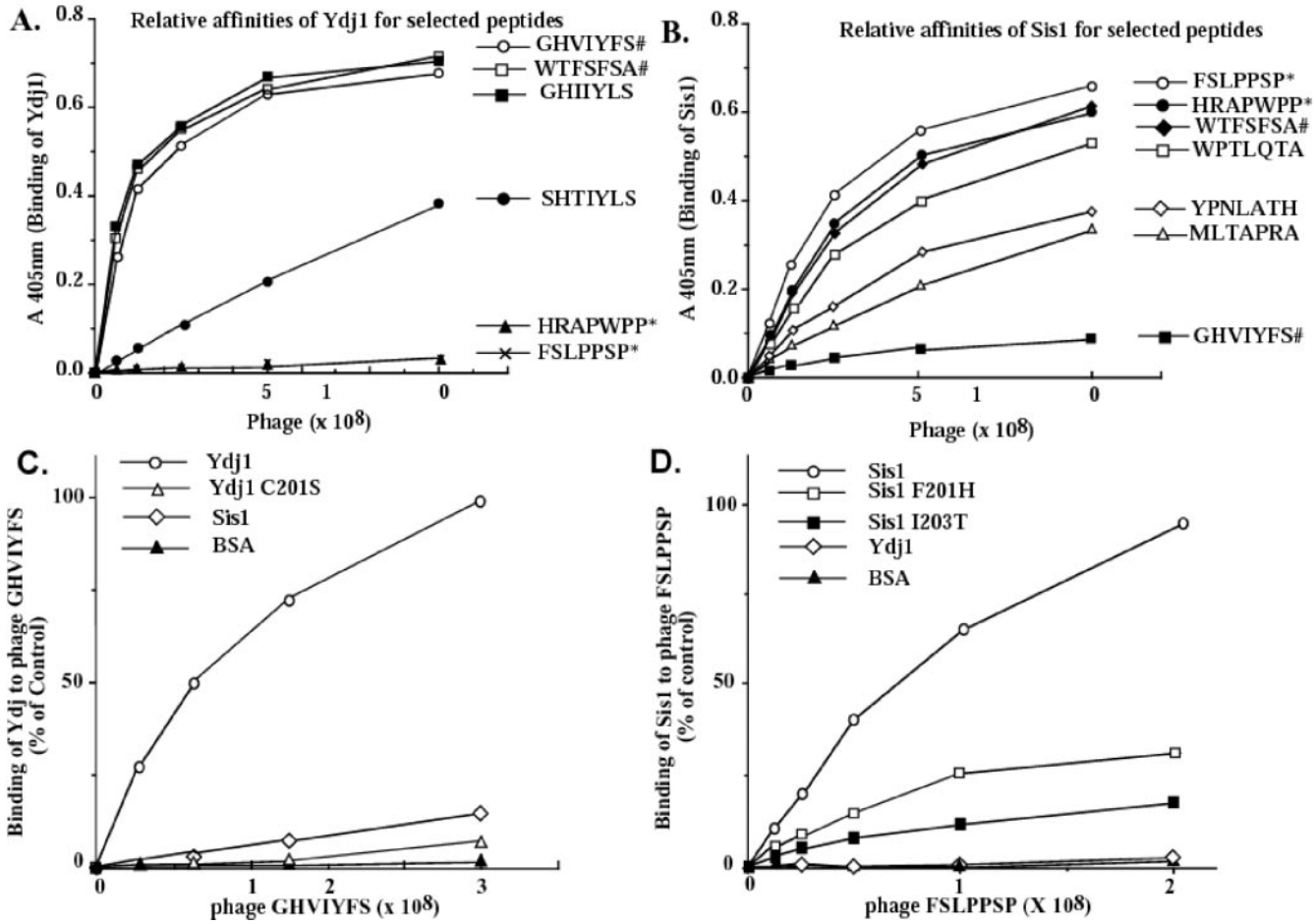

Figure 4. Binding of purified coliphage to Ydj1 and Sis1. (A) Binding curves that depict complex formation between immobilized Ydj1 and coliphage that display different peptides. (B) Binding curves that depict complex formation between immobilized Sis1 and coliphage that display different peptides. Ydj1 or Sis1 were immobilized on the walls of 96-well microtiter plates, and their ability to bind coliphage that display the indicated peptide was determined via ELISA with α-M13 mouse monoclonal antibody. The peptides marked with a # were selected by Ydj1 and were tested for interactions with both Ydj1 and Sis1. The peptides marked with an asterisk (*) were selected by Sis1 and were tested for interactions with both Sis1 and Ydj1. Values are expressed in the OD at 405 nm. (C) Mutation of the peptide binding domain of Ydj1 causes a defect in binding of coliphage GHVIYFS. The indicated Hsp40 (1 μg/50-μl reaction) was immobilized in the wells of a microtiter plate. Then, complex formation with purified coliphage GHVIYFS was determined. (D) Mutations in CTDI cause defects in complex formation between Sis1 and coliphage FSLPPSP. The indicated Hsp40 (1 μg/50-μl reaction) was immobilized in the wells of a 96-well microtiter plate. Then, complex formation with coliphage FSLPPSP was determined. Coliphage GHVIYFS and FSLPPSP were chosen as the respective substrates for Ydj1 and Sis1 in these studies because results in A and B demonstrated that they displayed peptides that were bound with the highest affinity by these respective chaperones. Values for binding are displayed as a percentage of the maximum OD reading observed when complex formation between wild-type Ydj1 or Sis1 was measured at the highest titer of coliphage shown.

both Ydj1 and Sis1, which suggests that Ydj1 and Sis1 have overlapping substrate specificity. However, Ydj1 and Sis1 selected peptides that exhibited differences in the arrangement and enrichment of different amino acids. Many of theYdj1 peptides had a hydrophobic core of three to four residues, but Sis1-selected peptides did not have such a core. P is highly abundant in the library and well represented in Sis1 peptides, but it was largely excluded from the Ydj1 substrates. In binding studies, Ydj1 preferred to bind coliphage-displayed peptides with a hydrophobic core over those that contained multiple P residues. Sis1, on the other hand, differed from Ydj1 and could bind P-enriched peptides with high relative binding affinity, but it exhibited low affinity for a coliphage that displayed a peptide with a hydrophobic core.

We realize that the data presented represent a small sampling of potential substrates of Ydj1 and Sis1, and we do not want to draw any conclusions about rules by which Ydj1 and Sis1 function to recognize a nonnative protein. However, the data presented are sufficient to support the conclusion that Ydj1 and Sis1 can bind peptides that are hydrophobic in nature and that these proteins exhibit selectivity in substrate binding.

YSY and SYS Exhibit a Switch in Substrate Specificity

Data presented in Figures 3 and 4 identified GHVIYFS and FSLPPSL as high-affinity substrates of Ydj1 and Sis1, respectively. Because Ydj1 C201S and Sis1 F201H exhibit defects in the binding of coliphage displayed peptides, the chaperone modules of Ydj1 and Sis1 are involved in substrate selection. However, the aforementioned mutations did not completely abolish substrate binding by Ydj1 and Sis1. Thus, we further investigated whether the chaperone modules of Ydj1 and

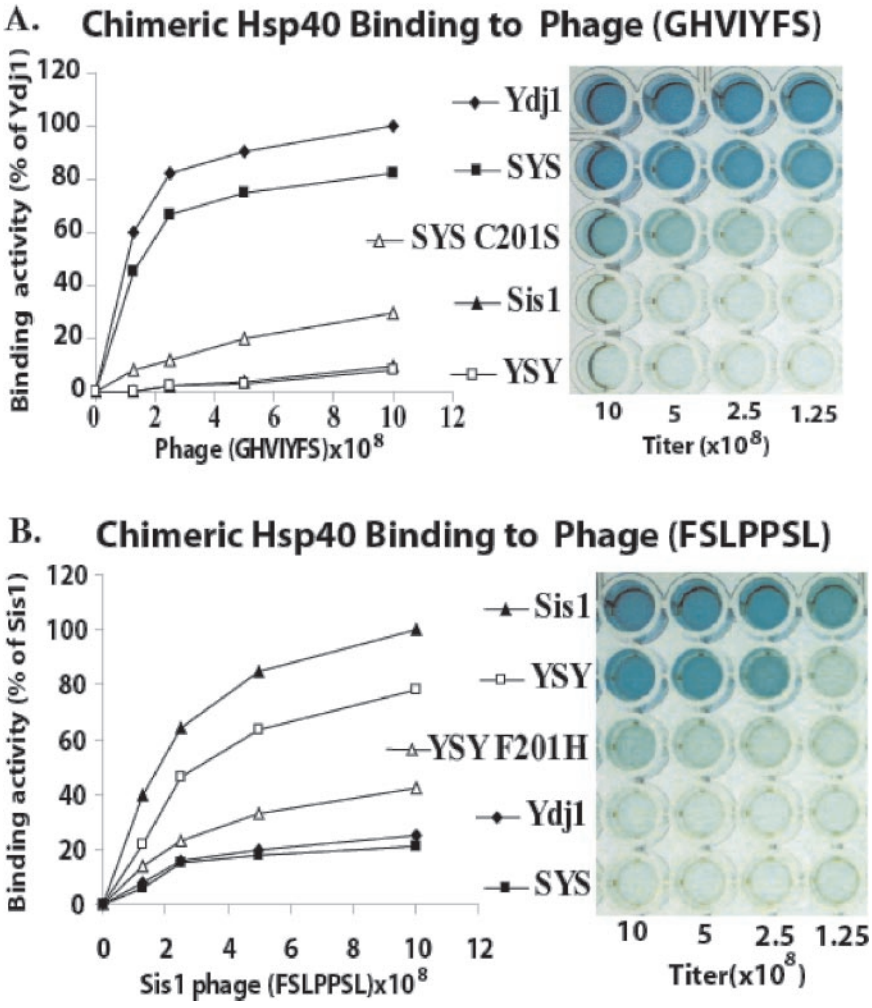

Figure 5. Analysis of the substrate specificity SYS and YSY. (A) Binding of chimeric Hsp40s to coliphage GHVIYFS. (B) Chimeric Hsp40 binding to coliphage FSLPPSL. The protein in a 100-μl aliquot of the indicated Hsp40 at a concentration of 100 nM was immobilized on the walls of 96-well microtiter plates, and serial dilutions of coliphage at the indicated titers were added to wells. The quantity of coliphage retained in the wells after extensive washing was then detected via ELISA with α-M13 antibody conjugated with horseradish peroxidase (Amersham Biosciences). Values are expressed as a percentage of the binding of coliphage GHVIYFS to Ydj1 and coliphage FSLPPSL to Sis1, respectively. (B) Photograph of the 96-well plate that was quantitated to generate the curves in A. The data shown are representative of three different experiments.

Sis1 control their substrate specificity by comparing the ability of YSY and SYS to bind coliphages GHVIYFS and FSLPPSL (Figure 5). SYS and Ydj1 were observed to bind coliphage GHVIYFS with high affinity (Figure 5A). Sis1 and YSY bound coliphage GHVIYFS with low affinity (Figure 5A). In addition, SYS C201S bound ~80% less coliphage GHVIYFS than Ydj1 and SYS.

Sis1 and YSY both recognized coliphage FSLPPSL with high affinity (Figure 5B). In contrast, complex formation between Ydj1 and SYS with coliphage FSLPPSL was low. YSY F201H bound ~60% less coliphage FSLPPSL than Sis1 and YSY. The defect in complex formation between YSY F201H and coliphage FSLPPSL was similar in extent to the defect in luciferase binding exhibited by YSY F201H (Figure 2).

The data presented in Figure 5 demonstrate that the selectivity of Ydj1 and Sis1 in binding coliphage-displayed peptides can be switched by swapping the chaperone modules located in the middle of these Hsp40s. Thus, the domains in Ydj1 and Sis1 that influence their luciferase folding activity also function to control Hsp40 substrate specificity.

YSY, but Not Ydj1, Can Support the Growth of a sis1Δ Strain

To test whether the chaperone modules of Ydj1 and Sis1 control the in vivo functions of these Hsp40s, we determined whether YSY or SYS could complement the lethal phenotype observed when the Sis1 gene is deleted from yeast strain W303A (Luke *et al*., 1991). This sis1Δ strain was chosen for use in these studies because it is inviable and this phenotype cannot be complemented by Ydj1 (Luke *et al*., 1991). Because Sis1 is capable of complementing the slow growth phenotype of ydj1Δ strains (Luke *et al*., 1991), the ability of YSY and SYS to complement ydj1Δ was not examined. Different forms of Ydj1 and Sis1 that were expressed under the control of their own promoter were introduced into sis1Δ by counterselection on 5-fluoroorotic acid and then growth phenotypes were monitored (Figure 6). As shown previously, Ydj1 was not able to support growth of sis1Δ (Figure 6A). On the other hand, YSY exhibited a gain of function and was able to support growth of sis1Δ. Therefore, the substitution of the G/M region and CTDI of Sis1 for the ZFLR of Ydj1 is sufficient to allow YSY to carry out the essential functions of Sis1. However, we also observed that SYS was capable of supporting the growth of sis1Δ. The chaperone modules located within YSY and SYS were demonstrated to be important for the in vivo functions of these chimeric Hsp40s because strains harboring YSY C201S and YSY F201H exhibited temperature sensitive defects in growth (Figure 6B). Western blot analysis of extracts from the respective strains with α-Sis1 sera demonstrated that all of the Ydj1 and Sis1 chimeras accumulated to a similar steady state level (Figure 6C). However, we did detect slightly less YSY and

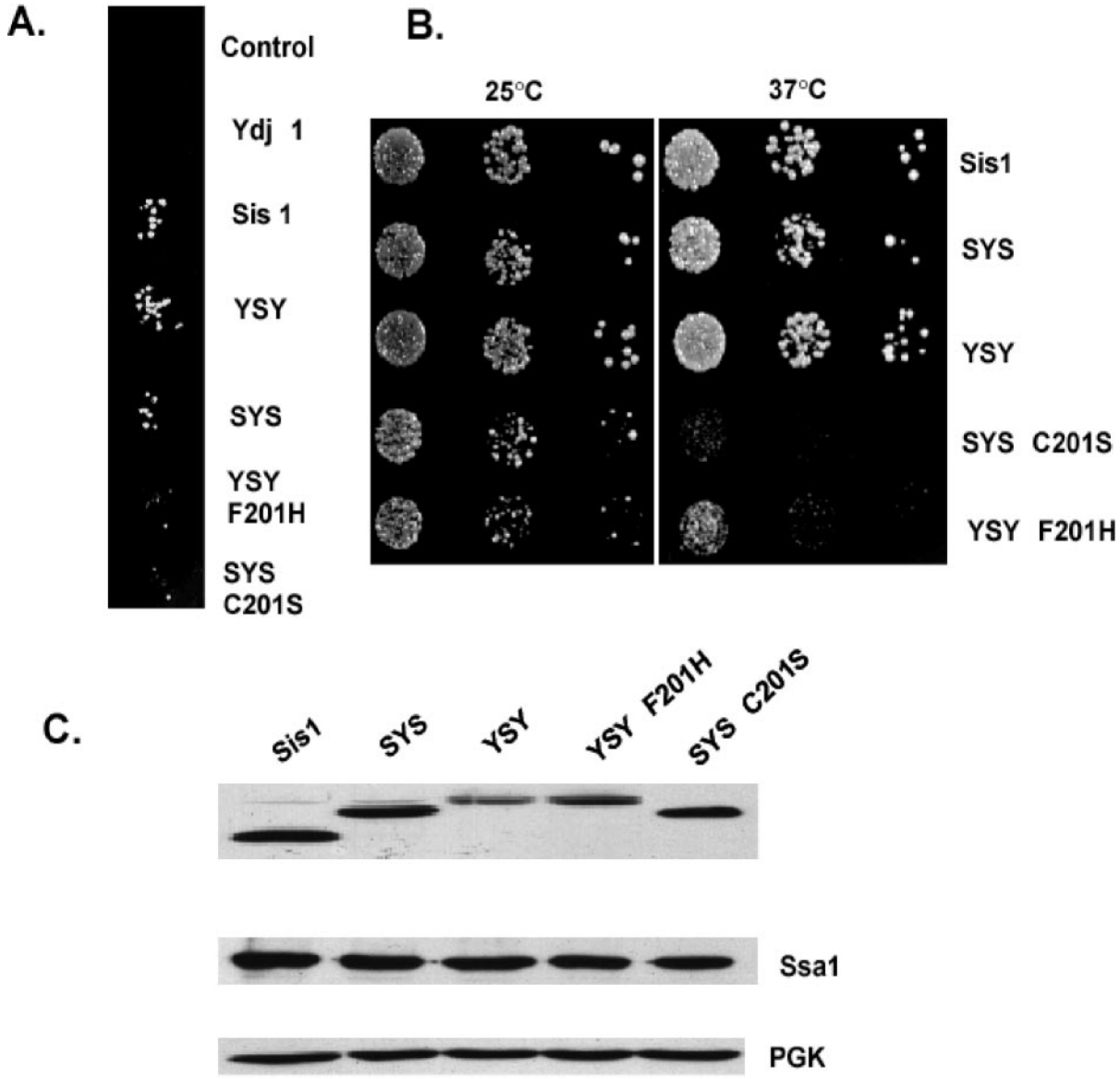

Figure 6. Complementation of growth defects in Δsis1 strain by YSY and SYS. (A) Growth of a sis1 Δ strain that harbors the indicated Hsp40 on 5-FOA. Δsis1 that harbored pRS316-Sis1 to maintain its viability was transformed with versions of pRS315 that contained the gene for the indicated Hsp40s. Transformants were selected and then grown in SD-selective liquid media at 30°C for 2 d. To counterselect for the pRS316-Sis1 plasmid in the sis1Δ, 7-μl aliquots of cells that were obtained from a culture grown to 0.1 OD at 600 nm were spotted onto SD plates that were supplemented with 5-FOA, adenine, tryptophan, and uracil (see MATERIALS AND METHODS for details). Plates were incubated for 3 d at 30°C and then photographed. (B) Growth of the sis1Δ strains on YPD. Sis1Δ strains that contained the indicated Hsp40 were cultured in SD-selective liquid media to an OD 600 of 0.1 and then 7-μl aliquots of 10-fold serially diluted cultures were spotted onto YPD plates. Plates were cultured for 2 d at 25°C or 37°C, respectively, and then photographed. (C) Western blot analysis of Hsp40 protein expression. The indicated yeast strains were cultured in YPD and then cell extracts were prepared by lysis with glass beads. Proteins in cleared extracts (50 μg) were resolved on a 12.5% SDS-PAGE gel and then transferred to a nitrocellulose membrane. Hsp40s present in the cell extract were detected with rabbit Sis1 α-serum. The level of Ssa1 expression was detected with a rabbit α-Ssa1 and these data are shown in the middle penal. The level of phosphoglycerate kinase (PGK) was probed with rabbit α-PGK sera, and these data are shown in the bottom panel.

YSYF201H. This result seems to have occurred because the majority of these proteins are constructed from Ydj1, and some of the epitopes recognized by the α-Sis1 sera were missing in YSY and YSY F201H.

The data presented in Figure 6 demonstrate chimeric Hsp40s YSY and SYS can function in vivo. The observation that YSY exhibits a gain of function and complements growth defects observed in sis1Δ strains suggest that the chaperone module of Sis1 is sufficient to specify the in vivo functions of Hsp40s.

However, it also seems that there are other regions within Ydj1 and Sis1 that function with the protein modules under study to help specify Hsp40 function. This seems to be the case because, although Ydj1 could not substitute for Sis1, SYS was able to maintain the viability of sis1Δ. Complementation studies with fragments of Ydj1 and Sis1 suggest that

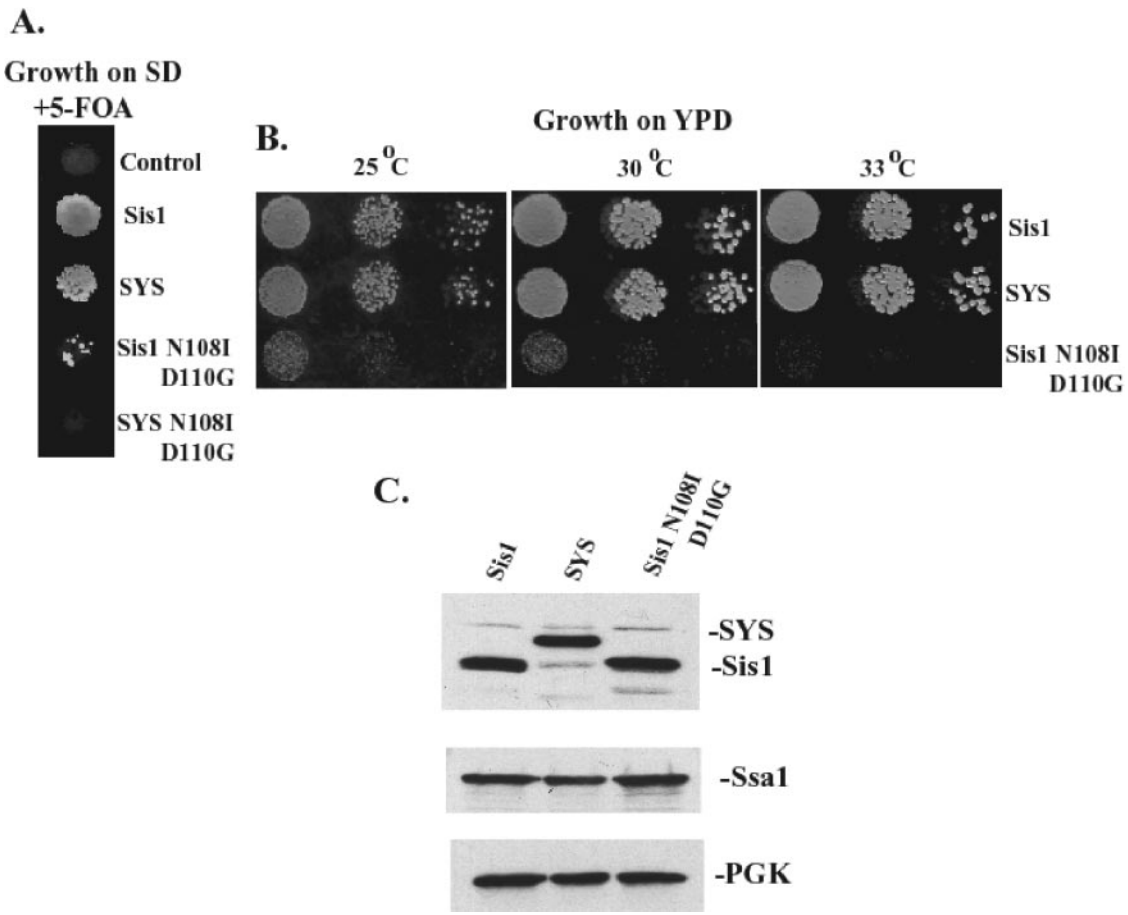

Figure 7. Mutations in the Sis1 G/F region prevent SYS from complementing growth defects in sis1Δ. (A) Complementation of sis1Δ by Sis1 G/F domain mutants. Yeast strain SL001 was transformed with the indicated plasmids and then spotted on 5-FOA–selective plates and incubated at 25°C for 6 d. (B) Growth of Sis1 G/F region mutants on YPD. After selection on 5-FOA, viable colonies were picked and cultured in synthetic dextrose media that was supplemented with Ade, Trp, and Ura liquid media. Aliquots (7 μl) of cultures grown to 0.1 OD_{600}/ml were serially diluted (1:10) and spotted on YPD plates. The plates were incubated for 3 d at indicated temperature. (C) Western blot analysis Sis1 and Ssa1 protein levels in cell extracts of the indicated strains. Blots were probed with antibodies against Sis1, Ssa1, and phosphoglycerate kinase (PGK).

the G/F-rich region of Sis1 and Ydj1 have unique features that enable them to help specify the in vivo functions of Hsp40s (Yan and Craig, 1999). The G/F region of Sis1 contains a 10-residue insert, GHAFSNEDAF, that corresponds to amino acids 102–112 that is not present in the G/F region of Ydj1 (Lopez *et al*., 2003). Mutation of N108 and D110 in the Sis1 G/F domain can alter the in vivo functions of Sis1(Lopez *et al*., 2003). Thus, we reasoned that SYS may be able to support the growth of sis1Δ because the Sis1 G/F region and the Ydj1 ZFLR functionally interact.

To test the aforementioned model the mutant proteins Sis1 N108I D110G and SYS N108I D110G were constructed and their ability to support the growth of sis1Δ was determined (Figure 7). Sis1 N108I D110G was able to support the viability of sis1Δ (Figure 7A), but sis1Δ strains that harbored this mutant exhibited a slow growth phenotype at 25°C and were in viable at 33°C (Figure 7B). In contrast, SYS N108I D110G was unable to support the growth of sis1Δ. These data further demonstrate that the G/F region of Sis1 is important for it to carry out its cellular functions. These data also imply that the Sis1 G/F domain functions with, or modifies the action of, the Ydj1 chaperone module to enable SYS to substitute for Sis1. Thus, the chaperone modules and the G/F regions of Ydj1 and Sis1 may cooperate in the specification of Hsp40 function.

Sis1 and YSY, but Not SYS Maintain [RNQ+]

To determine whether the chaperone domains of Ydj1 and Sis1 direct these chaperones to carry out specific cellular functions, we examined the ability of YSY and SYS to maintain the yeast prion [RNQ+]. [RNQ+] maintenance was chosen as an assay for these in vivo studies because Sis1 is known to bind Rnq1 (Luke *et al*., 1991) and somehow promote its assembly into prion fibrils (Sondheimer *et al*., 2001), but Ydj1 is unable to carry out this function (Lopez *et al*., 2003). Rnq1 is a 40-kDa soluble cytoplasmic protein that fractionates to the supernatant of cell extracts when it is in the nonprion form. But the prion form of Rnq1 ([RNQ+]) is assembled into pelletable aggregates (Sondheimer *et al*., 2001). [RNQ+] can be visualized in whole cells by monitoring the ability of Rnq1-GFP to assemble into punctate foci, with the nonprion form of Rnq1-GFP exhibiting a diffuse cytoplasmic staining pattern (Sondheimer *et al*., 2001).

We observed that Sis1 and YSY were both capable of maintaining [RNQ+], but SYS was unable to perform this

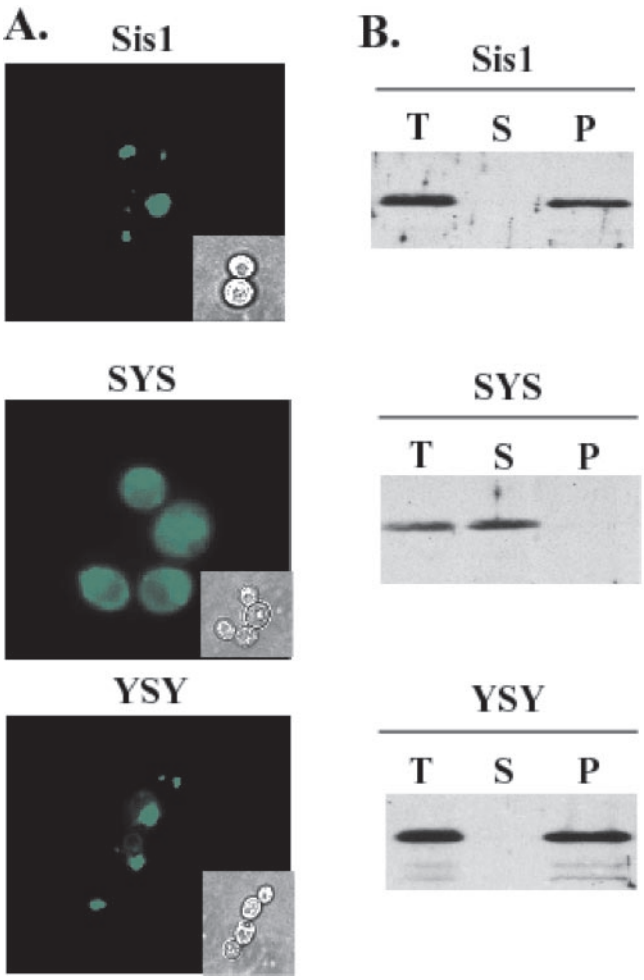

Figure 8. Maintenance of [RNQ+] by chimeric Hsp40s. (A) Fluorescence microscopy of Rnq1-GFP in sis1Δ-expressing Sis1, SYS, and YSY. The aggregation state of Rnq1-GFP aggregation state was monitored after induction with 50 μM $CuSO_4$ for 4 h. Cells exhibited are representative of >60% of those in the culture. The inserts are bright field images of cells. (B) Western blot analysis of the Rnq1 aggregation state. Cell lysates were prepared and fractionated under nondenaturing conditions by ultracentrifugation at 280,000 × *g* for 1 h. Equivalent aliquots of total (T) cell extract, supernatant (S), and pellet fractions were separated by SDS-PAGE and probed by immunoblot with Rnq1 polyclonal antibody.

function (Figure 8, A and B). To determine why YSY and SYS exhibited differences in [RNQ+] maintenance, complex formation between these chimeric Hsp40s and endogenous Rnq1 was examined (Figure 9A). Western blot analysis of cell extracts demonstrated that Rnq1 was present in total cell extracts at similar levels in sis1Δ strains that harbored SIS1, SYS, or YSY (Figure 9A). However, when these Hsp40s (Figure 9B) were immunoprecipitated from cell extracts, with α-Sis1 sera under native buffer conditions, Rnq1 was only observed to coimmunoprecipitate with Sis1 and YSY (Figure 9A). Because SYS could not maintain [RNQ1+], and although it was readily immunoprecipitated from cells extracts (Figure 8B), it did not coprecipitate Rnq1. These data indicate that the Sis1 chaperone domain enables Sis1 to bind Rnq1 and maintain [RNQ+]. These data are consistent with the interpretation that the centrally located protein modules found in type I and type II Hsp40s help specify at least some of the in vivo functions of Ydj1 and Sis1.

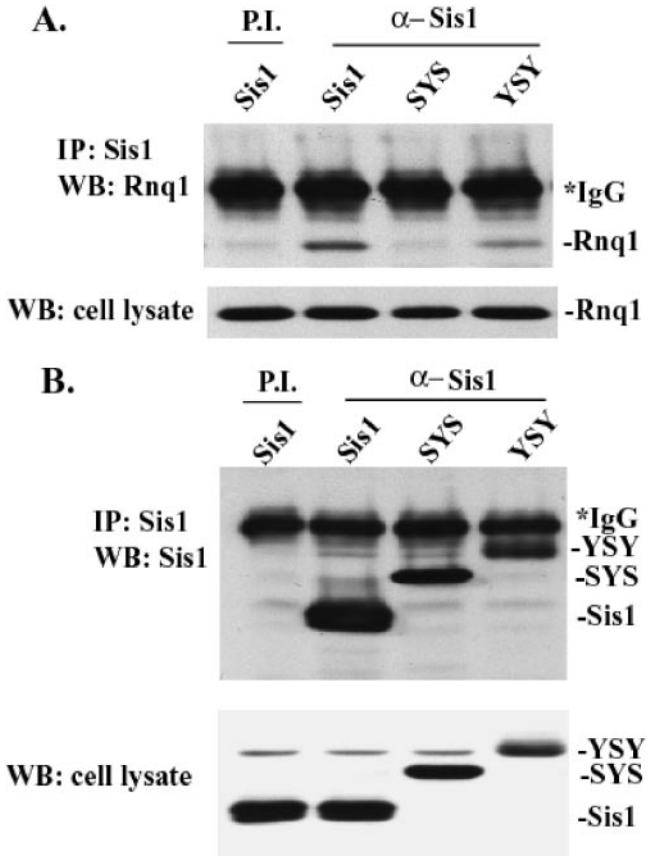

Figure 9. Sis1 chaperone module is required for Sis1 to coimmunoprecipitate with Rnq1. (A) Coimmunoprecipitation of Rnq1 with chimeric Hsp40s. Extracts from sis1Δ-expressing Sis1, SYS, and YSY were prepared under nondenaturing conditions and Sis1 was immunoprecipitated from cell extracts. The presence of Rnq1 in the immunoprecipitates was then detected by Western blot with α-Rnq1 sera. The amount of Rnq1 in total cell extracts was also probed by Western blot, and these data are presented in the bottom panel. (B) Relative quantities of Sis1, SYS, and YSY that were immunoprecipitated from cell extracts. Western blots of immunoprecipitates generated for A were reprobed with α-Sis1 sera to detect the quantity of Sis1, SYS, and YSY that was immunoprecipitated. The bottom panel represents a Western blot of total cell extract probed with α-Sis1 sera. P.I. denotes control immunoprecipitations carried out with Sis1 preimmune (P.I.) sera. * IgG denotes the position of IgG in the sera that was used for the immunoprecipitations that is present on membranes and cross reacts with the second antibody utilized to develop these Western blots.

DISCUSSION

Herein, we present experimental data that supports the interpretation that conserved protein modules located within the middle of Ydj1 and Sis1 control the functional specificity type I and type II Hsp40s. The chaperone modules of Ydj1 and Sis1 were demonstrated to be interchangeable and functioned to control the protein folding activity, substrate binding specificity and in vivo function of these different Hsp40s. Ydj1 and Sis1 could both bind phage displayed peptides that were enriched in aromatic and hydrophobic amino acids, but yet each exhibited differences in selectivity. These data suggest that differences in the ability of type I and type II Hsp40s to recognize aspects of nonnative protein structure contribute to their ability to specify Hsp70 action.

We present four lines of experimental evidence, which support the notion that type I and type II Hsp40s contain exchangeable protein modules that contribute to the specification the cellular functions of Ydj1 and Sis1. First, results obtained with purified YSY and SYS demonstrated that the protein modules located in the middle of Ydj1 and Sis1 control the luciferase folding activity of Hsp70 Ssa1. Second, compared with Ydj1 and Sis1, the substrate specificity of SYS and SYS was switched. Third, YSY exhibited a gain of function and could complement the lethal phenotype of sis1Δ strains, whereas Ydj1 could not. Fourth, Sis1 and YSY can support maintenance of [RNQ+], but SYS was incapable of facilitating this cellular event.

There is evidence in the literature that demonstrates that the G/F region found in type I and type II Hsp40s helps to specify their cellular functions (Yan and Craig, 1999; Sondheimer *et al.*, 2001; Lopez *et al.*, 2003). The data that we present are supportive of these previous studies and suggest that the exchangeable protein modules identified herein can cooperate with the G/F to perform at least some of Sis1's and Ydj1's cellular functions. This model is supported by the observation that mutation of the Sis1 G/F domain negates the ability of SYS to support the viability of sis1Δ. Therefore, the Sis1 G/F domain seems able to modify the action of the Ydj1 chaperone module and allow it to productively interact with essential Sis1 substrates and thereby maintain sis1Δ viability. However, because SYS was unable to bind Rnq1 or support [RNQ+] maintenance, but YSY could perform this function, the G/F region is not always sufficient to specify Sis1's cellular functions.

The exact mechanism by which the chaperone modules of Ydj1 and Sis1 contribute to the specification of their functions is not entirely clear. However, differences in the ability of the Hsp40 chaperone modules to interact with nonnative proteins seem to play a role in functional specification. For example, Ydj1 and Sis1 exhibited differences in substrate specificity that switched in YSY and SYS. Second, the ability of Sis1 and YSY, and inability of SYS, to maintain [RNQ+] correlated with their capability to form coimmunoprecipitable complexes with Rnq1. These data are consistent with the notion that the nature of the chaperone module present within an Hsp40 controls its ability to form productive complexes with polypeptide substrates.

A goal of this study was to determine why the Ydj1:Ssa1 chaperone pair refolds luciferase with severalfold greater efficiency than the Sis1:Ssa1 pair (Lu and Cyr, 1998b). Two observations made with the chimeric Hsp40s SYS and YSY establish that the chaperone domains of Sis1 and Ydj1 control the luciferase folding activity of these Hsp40s. First, SYS and YSY could refold denatured luciferase with the same efficiency as Sis1 and Ydj1, respectively. Second, the swapped chaperone module mutants SYS C201S and YSY F201H exhibited severe defects in luciferase refolding. Because Ydj1 and Sis1 seem to bind denatured luciferase with the same efficiency, the mechanistic reason as to why the chaperone module of Ydj1 is superior to that of Sis1 in refolding denatured luciferase is not clear. However, it is likely that during the collapse of luciferase to the native state that folding intermediates expose different ensembles of hydrophobic surfaces. Because Ydj1 and Sis1 exhibit differences in substrate specificity, it is possible that the preferential recognition of a late-stage folding intermediate by Ydj1 would enable it to refold luciferase with higher efficiency that Sis1. An alternative possibility is that chaperone domains of Ydj1 and Sis1 influence luciferase refolding efficiency via an event that occurs after substrate postbinding. Nonetheless, the data presented with purified YSY and SYS support the interpretations made from in vivo studies that indicate that the protein modules located in the middle of Ydj1 and Sis1 exert control over Hsp70 function.

Results from the phage peptide display studies presented represent the first analysis of the substrate specificity of Ydj1 and Sis1. Ydj1 and Sis1 selected sets of peptides from a 7meric coliphage peptide display library that were enriched in aromatic and bulky hydrophobic amino acids. However, the groups of peptides that were selected by Ydj1 and Sis1 exhibited differences in the enrichment of specific amino acids. The majority of the coliphage selected by Ydj1 had a hydrophobic core of three to four residues, but the Sis1-selected peptides did not have a hydrophobic core. These collective data demonstrate that the polypeptide binding domains of Ydj1 and Sis1 prefer to bind hydrophobic regions exposed by nonnative proteins and may have some overlapping substrate specificity, but yet are selective.

Because Ydj1 and DnaJ are both type I Hsp40s these chaperones would be expected to exhibit similar substrate selectivity. When our data are compared with results obtained by using DnaJ to screen 13meric cellulose-bound peptide arrays, this was found to be the case (Rudiger *et al.*, 2001). DnaJ-selected peptides that were enriched in the aromatic amino acids F, W, and Y, the large hydrophobics I and L and the polar residue H. Similar to Ydj1, the amino acids P and K were excluded from DnaJ peptides. Thus, DnaJ selects peptides that are similar in amino acid composition to Ydj1, but differ from Sis1 because it excludes P and K. Many of the phage-displayed peptides selected by Ydj1 were arranged in an X-H-X-I-Y-L-S pattern (X is any amino acid). The XIYL core of this motif closely mimics the hydrophobic core of I, Y, and L that is found in the consensus DnaJ binding sequence (Rudiger *et al.*, 2001). In addition, peptides recognized by Ydj1 are also similar in sequence to those from luciferase that are recognized by DnaJ (Rudiger *et al.*, 2001). Thus, DnaJ and Ydj1 share a conserved domain structure and substrate specificity.

An observed difference in Ydj1 and Sis1 peptide binding function was that Ydj1 disfavored peptides that contained P residues and exhibited low affinity for P-enriched peptides selected by Sis1. P is well represented in the coliphage library used, and many Sis1-selected peptides contained two or more P residues. The peptide binding groove of Sis1 contains two depressions that seem capable of making contacts with the side chains amino acids that are exposed on the surface of nonnative proteins (Lee *et al.*, 2002), and one of these depressions is known to accommodate P (Sha *et al.*, 2000). Thus, it seems that type II, but not type I Hsp40s, bind regions of nonnative proteins that contain P residues. This is interesting because Type I Hsp40s and Hsp70 have been shown to select regions of proteins that form β-sheets and disfavor α-helical regions (Rudiger *et al.*, 1997). Because P disrupts β-structure, these data suggest that type I and type II Hsp40s bind different regions within nonnative proteins. This suggested difference polypeptide binding activity might explain why Ydj1 and Sis1 exhibit differences in luciferase folding activity.

What enables Ydj1 and Sis1 to select different peptides from coliphage peptide display libraries? The data presented can be interpreted to suggest that type I and type II Hsp40s contain polypeptide binding grooves that have different restrictions on the side chains of amino acids that they accommodate. This mechanistic difference between Ydj1 and Sis1 is likely to occur because the polypeptide binding domains of these proteins are structurally distinct. The complete structure of the Type I Hsp40 peptide binding domain is not known. The NMR solution structure of a 79-amino

acid residue fragment from DnaJ that corresponds to the ZFLR depicts this domain to have a novel fold with an overall V-shaped extended β-hairpin topology (Martinez-Yamout *et al*., 2000). Such a structure is not found in type II Hsp40 proteins. The crystal structure of Sis1 (171–352) demonstrates that CTDI, which has replaced the ZFLR, is constructed from a series of β-strands that assemble into a barrel-like structure that contains a shallow peptide binding groove (Sha *et al*., 2000). Structural information that describes how Hsp40s make contacts with nonnative proteins is now required to uncover the mechanism for substrate binding by Ydj1 and Sis1.

It has been suggested that the Hsp40 C-terminal dimerization domain, which was identified in the Sis1 (171–352) structure, functions in polypeptide binding by type I and type II Hsp40s (Rudiger *et al*., 2001). However, YSY and SYS did not contain alterations in their C termini, but when compared with Ydj1 and Sis1, their substrate specificity and luciferase folding activity was switched. In addition, Ydj1 and Sis1 are both isolated from yeast cells as functional dimers, which means that the hydrophobic surfaces on the dimerization domain are not likely to be exposed to solvent and thus rendered unavailable for substrate binding. Thus, the dimerization domain of Hsp40s does not seem to play a major role in substrate binding.

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