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In Vivo Bipartite Interaction Between the Hsp40 Sis1 and Hsp70 in *Saccharomyces cerevisiae*

Rebecca Aron,*,†,1 Nelson Lopez,*,‡,1,2 William Walter,* Elizabeth A. Craig*,3 and Jill Johnson*,4

**Department of Biochemistry, †Graduate Program in Biomolecular Chemistry and ‡Graduate Program in Bacteriology, University of Wisconsin, Madison, Wisconsin 53706*

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ABSTRACT

The essential Hsp40, Sis1, is a J-protein cochaperone for the Ssa class of Hsp70's of *Saccharomyces cerevisiae*. Sis1 is required for the maintenance of the prion [*RNQ*$^+$], as Sis1 lacking its 55-amino-acid glycine-rich region (G/F) does not maintain [*RNQ*$^+$]. We report that overexpression of Sis1ΔG/F in an otherwise wild-type strain had a negative effect on both cell growth and [*RNQ*$^+$] maintenance, while overexpression of wild-type Sis1 did not. Overexpression of the related Hsp40 Ydj1 lacking its G/F region did not cause inhibition of growth, indicating that this dominant effect of Sis1ΔG/F is not a characteristic shared by all Hsp40's. Analysis of small deletions within the *SIS1* G/F region indicated that the observed dominant effects were caused by the absence of sequences known to be important for Sis1's unique cellular functions. These inhibitory effects of Sis1ΔG/F were obviated by alterations in the N-terminal J-domain of Sis1 that affect interaction with Ssa's ATPase domain. In addition, a genetic screen designed to isolate additional mutations that relieved these inhibitory effects identified two residues in Sis1's carboxy-terminal domain. These alterations disrupted the interaction of Sis1 with the 10-kD carboxy-terminal regulatory domain of Ssa1, indicating that Sis1 has a bipartite interaction with Ssa *in vivo*.

IT is well established that molecular chaperones function in a wide range of cellular processes. For example, chaperones assist the folding of other proteins by preventing their aggregation and, in some cases, by resolubilizing protein aggregates. In addition, a role for molecular chaperones in modulating the state of prions, proteins that have the unusual ability to exist in distinct and heritable conformations, has begun to emerge (Wickner *et al.* 1999; Serio *et al.* 2000); the stability of these prion states allows for the faithful transmission of prions during cell division.

While a number of classes of molecular chaperones exist, Hsp70 and J-type chaperones are among the most conserved, being present in nearly all organisms. Hsp70's and J-proteins function together (Bukau and Horwich 1998). Neither Hsp70 nor J-proteins alone are capable of promoting the refolding of denatured luciferase *in vitro*, but together they can cooperate to efficiently effect refolding. Hsp70's bind unfolded or partially unfolded polypeptides in an ATP-regulated cycle. Whereas the central 18-kD domain binds short hydrophobic stretches of amino acids, the highly conserved N-terminal 44-kD domain regulates this binding of substrate polypeptides through its interaction with adenine nucleotides. Therefore, Hsp70 proteins have a two-state conformation. When an ADP molecule is bound to the nucleotide-binding site, the Hsp70 exhibits stable peptide binding; when ATP is bound, this interaction is relatively unstable.

On the basis of biochemical analyses, J-proteins are thought to facilitate Hsp70 action in two ways (Young *et al.* 2004). First, they stimulate ATP hydrolysis, promoting a stable interaction between Hsp70 and unfolded proteins. Second, some J-proteins bind unfolded polypeptide substrates and are able to prevent their aggregation independently of Hsp70 action. Therefore, according to the current model of the cycle of Hsp70 and J-protein action, a J-protein first binds unfolded protein substrate and then transfers it to Hsp70, simultaneously stimulating the Hsp70 ATPase activity and thus stabilizing the Hsp70-unfolded protein interaction.

Multiple J-proteins exist in both prokaryotic and eukaryotic cells. The highly conserved J-domain interacts with the Hsp70 ATPase domain in an ATP-dependent manner (Bukau and Horwich 1998). A major subset of J-proteins called Hsp40's (Cheetham and Caplan 1998) has a N-terminal J-domain, followed by a region rich in glycine residues, which in turn is followed by a domain that binds unfolded polypeptides. The Hsp40

[1]These authors contributed equally to this work.

[2]*Present address:* Process Development Department, Amgen, Juncos, Puerto Rico 00777-4060.

[3]*Corresponding author:* Department of Biochemistry, 441E Biochemistry Addition, 433 Babcock Dr., University of Wisconsin, Madison, WI 53706. E-mail: ecraig@wisc.edu

[4]*Present address:* Department of Microbiology, Molecular Biology and Biochemistry, University of Idaho, Moscow, ID 83844.

Sis1, the subject of this report, is the J-protein partner of members of the Ssa family of Hsp70's (Ssa1-4) (Lu and Cyr 1998). Sis1 contains an extended glycine-rich region compared to other Hsp40's, such as *Escherichia coli* DnaJ or yeast Ydj1. The first 55 amino acids of this region of Sis1 are also rich in phenylalanines (G/F region); the last 49 amino acids are rich in methionine residues (G/M). The carboxy-terminal 181 amino acids of Sis1 contain the proposed polypeptide binding site (domain I), a domain of unknown function (domain II), and a dimerization domain (Lu and Cyr 1998; Sha *et al.* 2000; Lee *et al.* 2002; Li *et al.* 2003). In addition to the J-domain:ATPase domain interaction, an interaction between the carboxy-terminal region of Sis1 and the C-terminal 10-kD domain of Hsp70 has been detected (Demand *et al.* 1998; Qian *et al.* 2002). In the cases of Ssa1 and Sis1, the interaction requires the last four amino acids of Ssa1, but the *in vivo* significance of this interaction between the C termini of the two proteins is unknown.

Sis1 is critical for maintenance of the prion form of the protein Rnq1 (Sondheimer *et al.* 2001; Fan *et al.* 2004; N. Lopez, R. Aron, W. Walter, E. Craig and J. Johnson, unpublished results). Like other prion-forming proteins, Rnq1 exists in different states: a soluble form, [*rnq*$^-$], and an aggregated prion form, [*RNQ*$^+$]. Sis1 lacking the G/F region (Sis1ΔG/F) is unable to maintain [*RNQ*$^+$] (Sondheimer *et al.* 2001). The role of Sis1 in maintenance of [*RNQ*$^+$] is not related to its essentiality (Zhong and Arndt 1993), as neither a deletion of the *RNQ1* gene nor the presence of [*RNQ*$^+$] causes an obvious phenotype (Sondheimer and Lindquist 2000). Sis1 plays multiple roles in the cell, and evidence points to a role in the initiation of translation as an essential cellular Sis1 function (Zhong and Arndt 1993).

Sis1 is functionally unique in that it performs roles that are not covered by related proteins. Overexpression of another cytosolic Hsp40, Ydj1, cannot restore wild-type growth of a *sis1* strain (Luke *et al.* 1991) nor is Ydj1 required for the maintenance of [*RNQ*$^+$] (Lopez *et al.* 2003). Surprisingly, the specificity of Sis1 function resides in the glycine-rich region (Yan and Craig 1999). The C-terminal sequences extending beyond the glycine-rich regions, including the polypeptide-binding domain, are essential neither for cell viability nor for maintaining Rnq1 in an aggregated state. However, they play some role as cells expressing only the J-domain and the G/F region of Sis1 grow somewhat more slowly than wild-type cells, and although Rnq1 is maintained in a prion state, smaller aggregates are observed (Sondheimer *et al.* 2001).

Because of the critical nature of the G/F region of Sis1, we began an analysis of *sis1ΔG/F*. We found that *sis1ΔG/F* had a negative effect on both prion maintenance and cell growth when overexpressed in wild-type cells. However, these negative effects were suppressed by mutations causing single amino acid alterations of Sis1 that disrupt interaction with either the ATPase domain or the 10-kD regulatory regions of Ssa1. Our results suggest that Sis1 has a bipartite interaction with Ssa1 *in vivo*.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions: All genetic experiments were carried out in the W303 background, mainly utilizing PJ51-3a (a *trp1-1 ura3-1 leu2-3,112 his3-11,15 ade2-1 can1-100 GAL2*$^+$ *met2-Δ1 lys2-Δ*). For testing the function of *sis1* mutants in the absence of wild-type *SIS1*, WY26 (α *trp1-1 ura3-1 leu2-3,112 his3-11,15 ade2-1 can1-100 GAL2*$^+$ *met2-Δ1 lys2-Δ2 sis1::LEU2/YCP50-SIS1*) (Yan and Craig 1999) was transformed with pRS314 (Sikorski and Hieter 1989) carrying *SIS1* mutants. Colonies having lost wild-type *SIS1* were selected on plates containing 5-fluoroorotic acid (5-FOA). WY12 (α *his3-11, 3-15 leu2-3,2-112 ura3-52 trp1-Δ1 lys2 Δpep4::HIS3 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2*) (Pfund *et al.* 2001) and Y1121 (α *Δpep4::HIS3 Δsis1::LEU2/YCP50-SIS1 leu2-3,112 ura3-52 trp1-Δ1 his3-11,15 lys1 lys2*) were used for protein purification after transformation with the plasmids indicated below.

Strains were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) or minimal media lacking specific amino acids (0.67% yeast nitrogen base without amino acids, 2% dextrose, supplemented with all required amino acids). To analyze cell growth, serial dilutions of logarithmically growing cells were spotted onto YPD or selective minimal media.

Plasmids pYW65 (pRS314-*SIS1*), pYW116 (pRS314-*sis1ΔG/F*), pYW118 (pRS314-*sis1-H34Q*), and p316-*RNQ1-GFP* have been described elsewhere (Yan and Craig 1999; Sondheimer and Lindquist 2000). Other plasmids described below were constructed by standard molecular techniques.

Rnq1 prion analysis: Centrifugation assays to determine the aggregation state of Rnq1 were performed as described (Sondheimer *et al.* 2001). For fluorescence microscopy, cells were transformed with a copy of *RNQ1-GFP* regulated by the *CUP1* promoter (Sondheimer and Lindquist 2000). Cells were grown at 30° and visualized during midlog phase after induction with 50 μM $CuSO_4$. Unfixed cells were visualized in a fluorescence microscope Zeiss Axioplan 2 (Zeiss, Oberkochen, Germany). Digital images were captured using the QED software (QED Imaging, Pittsburgh).

Protein purification: Ssa1 was purified from WY12 transformed with plasmid p416TEFHIS-*SSA1* using metal chelate affinity chromatography as described (Pfund *et al.* 2001). Purified proteins were dialyzed against a standard Tris buffer (20 mM Tris, pH 7.5, 20 mM NaCl,10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 10% glycerol). Wild-type and mutant Sis1 proteins with an amino-terminal histidine-tag were generated by cloning into the pRSETB vector (Invitrogen, Carlsbad, CA). For expression in yeast, this construct was placed under control of the glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter in p414GPD or related vectors (Mumberg *et al.* 1995) and transformed into Y1121. To ensure that the His-tagged Sis1 was the only Sis1 present, transformants were transferred to media containing 5-FOA to counterselect for the Ycp50-*SIS1* plasmid. Due to reduced viability of cells expressing only Sis1Δ170-255 (data not shown), an additional untagged version of Sis1 lacking the dimerization domain (Sha *et al.* 2000) was present to improve cell growth. Purification was done as described for Ssa1.

Genetic analysis: To obtain consistent overexpression levels, wild-type *SIS1*, *sis1ΔG/F*, and *sis1ΔG/F-H34Q* (not encoding the amino-terminal histidine tag) were moved into the centromeric 414GPD and 415GPD vectors (Mumberg *et al.* 1995). PJ51-3A was transformed with these plasmids using standard yeast methods (Gietz *et al.* 1995). Immunoblot analysis (not shown) determined that wild-type cells containing 414GPD-

SIS1 expressed four to five times the amount of Sis1 produced from the chromosomal copy of *SIS1* alone. 414GPD plus 415GPD (or 416GPD) was used to achieve the same level of overexpression of variants of Sis1 having deletions of the G/F region. In all experiments reported, the overexpression of Sis1 or Sis1 variants was at least four or three times, respectively, the level produced from the chromosomal copy of *SIS1* alone. For overexpression analysis of Ydj1 and Ydj1ΔGF, pRS317-Ydj1 or Ydj1ΔGF was cotransformed with pRS424-Ydj1 or Ydj1ΔGF into PJ51-3A cells. Overexpression of approximately two- and fourfold for Ydj1 and Ydj1ΔGF, respectively, relative to endogenous Ydj1, was determined by immunoblot analysis.

A plasmid library containing random mutations in codons 158–352 of Sis1ΔG/F was generated using error-prone PCR and transformed into a strain that grew well unless *sis1ΔG/F* was overexpressed. Two colonies arose despite producing full-length Sis1ΔG/F. Plasmid DNA from these colonies was isolated and the mutations were identified as *sis1ΔG/F-L268P* and *sis1ΔG/F-G315D*. These mutations were subsequently moved into the p414GPD and p415GPD vectors for overexpression and purification. In addition, the L268P and G315D mutations were separated from the ΔG/F mutation using internal restriction sites and moved into full-length *SIS1* for purposes of protein purification.

Immunoblot analysis: Immunoblots were performed as described (Sondheimer *et al.* 2001; Lopez *et al.* 2003) using polyclonal antibodies developed in the Craig laboratory against full-length Sis1 (66932) or the first 185 residues of Rnq1 (1121). Samples were separated by SDS-PAGE and analyzed by immunoblot using chemiluminescence detection (New England Nuclear, Boston).

Substrate-binding ELISA assays: Firefly luciferase (Promega, Madison, WI) or Rnq1 was denatured for 1 hr at 25° in 3 M guanidine HCl, 25 mM HEPES, pH 7.5 (KOH), 50 mM KCl, 5 mM $MgCl_2$, and 5 mM DTT. Substrate was diluted in 0.1 M $NaHCO_3$ and bound to CoStar 3590 EIA plates (Corning, Corning, NY) at a concentration of 0.1 μg/well. Wells were washed with phosphate-buffered saline (PBS), blocked with PBS containing 0.5% fatty-acid-free bovine serum albumin (BSA) (Sigma, St. Louis), and washed with PBS containing 0.05% Tween 20 (PBST). Wild-type or mutant Sis1 was serially diluted in PBST + 0.5% BSA and incubated with substrate for 1 hr at room temperature. After extensive washing with PBST, a 1:20,000 dilution of polyclonal antisera against Sis1 was added and incubated for 1 hr. After additional washes, a 1:4000 dilution of donkey-anti-rabbit HRP-conjugated antiserum (Amersham, Piscataway, NJ) was added and incubated for 45 min. After washing, the reaction was developed according to the manufacturer's specifications with TMB peroxidase EIA substrate kit (Bio-Rad, Hercules, CA).

Ssa1 10-kD binding assays: Ten-kilodalton fragments of Ssa1 were expressed as GST fusion proteins in *E. coli* using pGEX-KG (Guan and Dixon 1991), purified on glutathione-agarose beads (Sigma), and released from GST by thrombin cleavage as described (Guan and Dixon 1991). The 10-kD fragment or BSA as a control was diluted into 0.1 M $NaHCO_3$ and 7 pmol was bound to each well of EIA plates. The Sis1-binding assay was performed as described for the substrate-binding assay.

Other assays: Single-turnover ATPase and luciferase refolding assays were performed essentially as described (Liu *et al.* 2001).

RESULTS

Sis1ΔG/F binds denatured substrates, but does not efficiently cooperate with Hsp70 to refold luciferase.

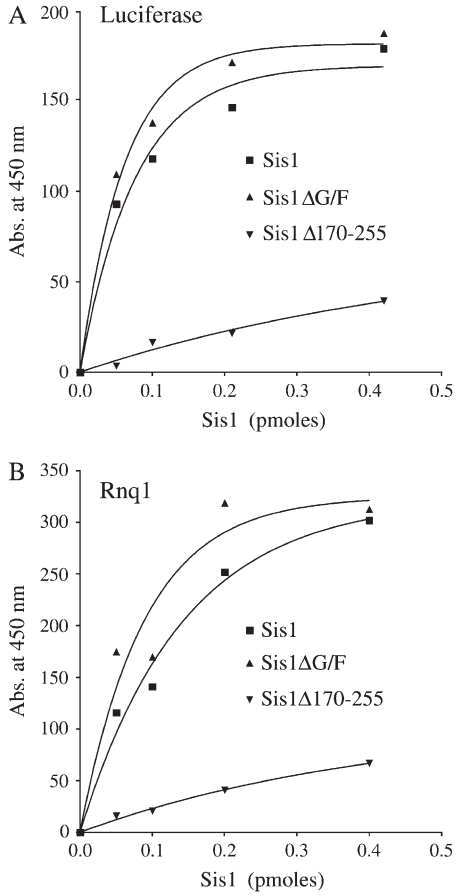

Figure 1.—Sis1ΔG/F binds denatured protein. (A and B) Binding of purified wild-type Sis1, Sis1ΔG/F, or Sis1Δ170-255 to denatured luciferase (A) or Rnq1 (B). Serial dilutions of purified Sis1 (4, 2, 1, 0.5, and 0 nM) were incubated with 100 ng of luciferase or Rnq1 immobilized in wells of a microtiter plate. Bound Sis1 was detected with antibody specific for Sis1.

Since Sis1ΔG/F is severely defective in the maintenance of [RNQ^+], we purified Sis1 and Sis1ΔG/F to compare their physical and functional interactions with unfolded protein substrates and with Ssa1. We first determined if Sis1ΔG/F had an altered interaction with unfolded proteins using a modified and sensitive ELISA assay previously used to study interactions between the *E. coli* Hsp40 DnaJ and substrates (Wawrzynow and Zylicz 1995). Chemically denatured luciferase (Figure 1A) or Rnq1 (Figure 1B) was immobilized in wells of microtiter plates. Increasing concentrations of wild-type or mutant Sis1 were added, and the amount of Sis1 retained in the wells after extensive washing determined using Sis1-specific antibodies. A Sis1 mutant protein lacking domain I (Sis1Δ170-255), the peptide-binding domain, was used as a control. As expected, Sis1Δ170-255 bound very poorly to denatured luciferase or Rnq1 compared to wild-type protein. However, binding of Sis1ΔG/F was indistinguishable from that of wild-type Sis1, indicating that it was not defective in substrate binding.

We next tested the ability of Sis1ΔG/F and Ssa1 to function together. First, we compared the ability of Sis1

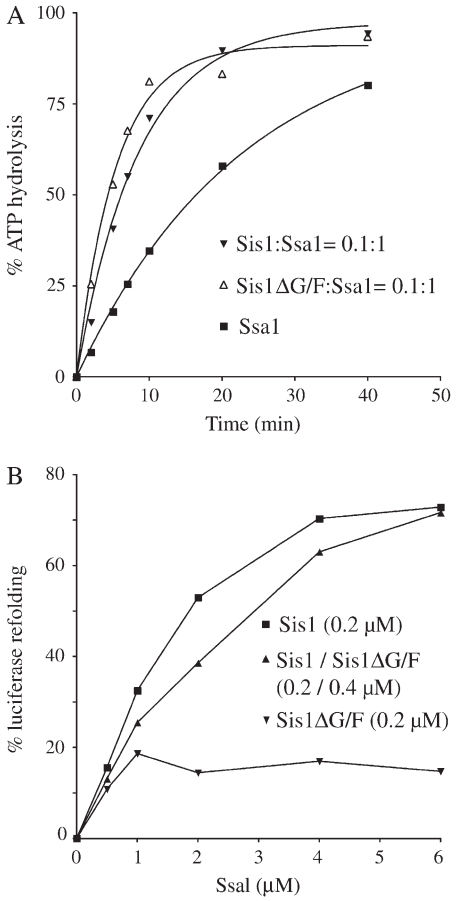

FIGURE 2.—Sis1ΔG/F is able to stimulate the ATPase of Ssa1 but is unable to cooperate in the refolding of denatured luciferase. (A) Stimulation of Ssa1 ATPase activity. A total of 0.2 μM Ssa1 in complex with radiolabeled [γ-^{32}P]ATP was incubated in the presence or absence of 0.02 μM Sis1 or Sis1ΔG/F. Fraction of ATP converted to ADP was determined at indicated times. (B) Refolding of denatured luciferase. Denatured luciferase was incubated with 0.2 μM Sis1 or Sis1ΔG/F in the presence of indicated amounts of Ssa1. Luciferase activity is expressed as percentage of activity prior to denaturation.

and Sis1ΔG/F to stimulate the ATPase activity of Ssa1, as typically Hsp40's stimulate the intrinsic ATPase activity of Hsp70's by increasing the rate of hydrolysis. We used a single-turnover assay in which preformed complexes between purified Ssa1 and [α-^{32}P]ATP were incubated in the absence of Sis1 or in the presence of wild-type Sis1 or Sis1ΔG/F. Over a range of concentrations we observed no substantial difference in the ability of Sis1 and Sis1ΔG/F to stimulate Ssa1 ATPase activity (Figure 2A) (data not shown).

Second, we tested the ability of Sis1 and Ssa1 to facilitate the refolding of denatured luciferase. As expected from previously published results (LU and CYR 1998), wild-type Sis1 could facilitate refolding of up to 75% of the denatured protein (Figure 2B). However, Sis1ΔG/F was compromised in its ability to refold luciferase over a range of Ssa1 concentrations. Hsp40's are thought to target substrates to Hsp70 by first binding them and then facilitating their transfer to Hsp70. The inability of Sis1ΔG/F to efficiently facilitate refolding of luciferase, even though it could bind unfolded polypeptide, suggested that the G/F region may be important for a productive interaction between Hsp40 and Hsp70.

To test whether the folding defect of Sis1ΔG/F was manifest in the presence of wild-type protein, we examined the effect of adding Sis1ΔG/F to a folding reaction containing wild-type Sis1 and Ssa1 (Figure 2B). Partial inhibition of folding occurred in the presence of a twofold excess of Sis1ΔG/F. For example, when 2 μM Ssa1 was used in the assay, Sis1ΔG/F had 33% of the activity of wild-type Sis1. A mixture of wild type and Sis1ΔG/F had 70% of the activity of wild type alone. Thus, *in vitro* Sis1ΔG/F is defective in chaperone activity and partially inhibits the ability of wild-type Sis1 to facilitate protein folding.

Sis1ΔG/F has a dominant negative effect on maintenance of [*RNQ*$^+$] and cell growth: The *in vitro* experiments described above indicate that Sis1ΔG/F is defective in functional interactions with Ssa1 and has an inhibitory effect on the ability of wild-type Sis1 to refold luciferase *in vitro*. Therefore we asked if Sis1ΔG/F also had dominant effects on the maintenance of [*RNQ*$^+$] or on cell growth. To analyze the physical state of Rnq1 in strains overexpressing Sis1ΔG/F, we used two assays. We assayed the sedimentation properties of Rnq1 in cell lysates and we monitored the distribution of Rnq1 in living cells, making use of a Rnq1-green fluorescent protein fusion (Rnq1-GFP). As controls we tested Δ*sis1* cells expressing wild-type *SIS1* or *sis1ΔG/F* from a centromeric plasmid. As previously reported (SONDHEIMER and LINDQUIST 2000; LOPEZ *et al.* 2003), all of the Rnq1 was present in the pellet fraction and fluorescence appeared in a single bright focus in the majority of cells, with the rest of the cell having a dark background, when cells expressing wild-type Sis1 were tested. In cells expressing Sis1ΔG/F, all the Rnq1 was in the soluble fraction and the fluorescence was diffuse (Figure 3A), very similar to that seen in wild-type cells that had been cured of the prion.

To test whether overexpression of Sis1ΔG/F has a detrimental effect on prion maintenance in the presence of wild-type Sis1, wild-type strains overexpressing either Sis1 or Sis1ΔG/F were assayed for the presence of [*RNQ*$^+$] by high-speed centrifugation of cell lysates. Fourfold overexpression of wild-type Sis1 did not modify the sedimentation properties of Sis1, as no detectable Sis1 was present in the supernatant fraction (Figure 3B, bottom). However, threefold overexpression of Sis1ΔG/F consistently resulted in 15–17% of Rnq1 being in the supernatant. This effect of Sis1ΔG/F is partially dominant, as all Rnq1 in extracts from cells expressing only Sis1ΔG/F is found in the supernatant fraction. In addition, overexpression of Sis1ΔG/F, but not of wild-type Sis1, in wild-type cells affected the distribution of Rnq1-GFP (Figure 3B, top). The majority of cells overexpressing Sis1ΔG/F in the presence of normal levels of Sis1 displayed a diffuse

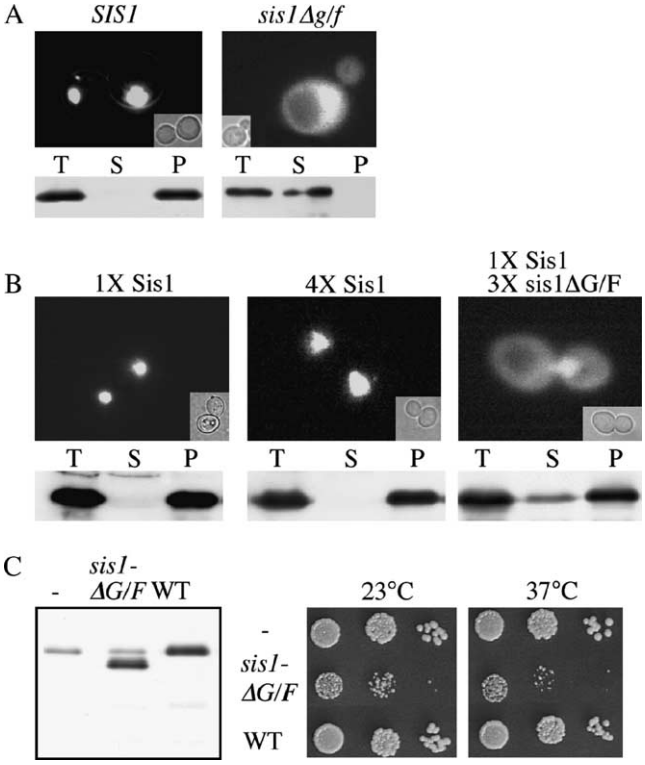

FIGURE 3.—Overexpression of Sis1ΔG/F in the presence of wild-type Sis1 is deleterious for maintenance of [*RNQ*$^+$] and cell growth. (A and B) Analysis of Rnq1. (Top) Cell lysates were separated by centrifugation and immunoblotted with antibody specific for Rnq1; total cell lysate (T), supernatant (S), and pellet (P) fractions. (Bottom) Fluorescence microscopy of Rnq1-GFP. Insets are the corresponding bright-field images. (A) Δ*sis1* cells expressing wild-type *SIS1* (*SIS1*) or *sis1ΔGF*. (B) Wild-type cells harboring plasmids: vector as a control (1× Sis1) or *SIS1* (4× Sis1) or *sis1ΔG/F* (1× Sis1 + 3× Sis1ΔG/F). (C, left) Equal amounts of lysates from wild-type cells (−), or cells overexpressing *sis1ΔG/F* (Sis1ΔG/F) or *SIS1* (4× Sis1) were separated by SDS-PAGE and immunoblotted with antibodies specific for Sis1. (C, right) Growth of serial dilutions of the same strains on selective medium after a 2-day incubation at 23° and 37°.

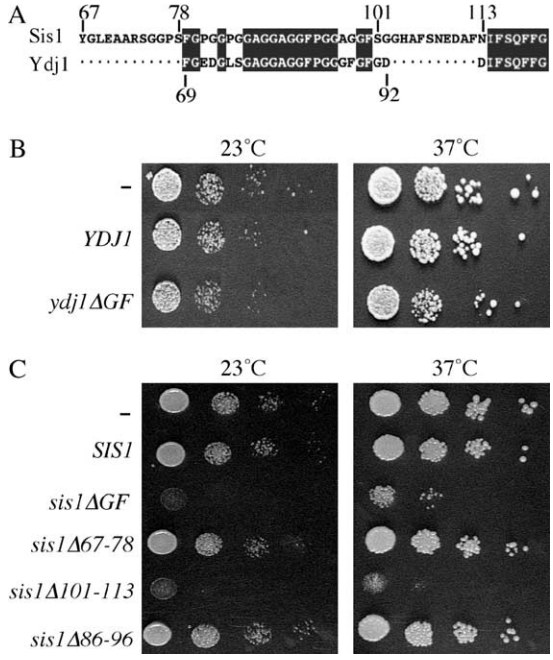

FIGURE 4.—Different effects of overexpression of Ydj1 and Sis1 lacking G/F sequences. (A) Comparison of the G/F region of Sis1 and Ydj1. Identical residues are boxed. (B and C) Wild-type cells harboring plasmids: vector as control (−) or vector containing the indicated genes. Tenfold serial dilutions of cells were spotted onto selective media and the plates were incubated at the indicated temperatures for 2 days.

pattern of fluorescence, very similar to that seen in a strain expressing only Sis1ΔG/F (Figure 3A). Therefore, both assays indicated a disruption in the maintenance of the prion [*RNQ*$^+$] when Sis1ΔG/F is present along with normal amounts of wild-type protein.

Although deletion of *RNQ1* has little or no effect on cell growth, Sis1 is essential for cell viability (LUKE *et al.* 1991). Therefore, we also evaluated whether overexpression of Sis1ΔG/F had any effects on growth rate. Threefold overproduction of Sis1ΔG/F in otherwise wild-type cells dramatically affected growth particularly at temperatures above and below the optimum of 30° (Figure 3C), while increased levels of wild-type Sis1 did not. Therefore, Sis1's ability to allow growth and maintain [*RNQ*$^+$] is sensitive to moderate overexpression of Sis1 lacking the G/F region.

Sis1 deleted for a 13-amino-acid segment of the G/F region has dominant effects: To better understand what sequences are causal in growth inhibition and prion maintenance, we analyzed additional deletion mutants. First, we tested another Hsp40 of the yeast cytosol, Ydj1. We asked if overexpression of the Hsp40 Ydj1 lacking its G/F region (Ydj1ΔG/F) had deleterious effects on growth relative to overexpression of wild-type Ydj1. Fourfold overexpression of Ydj1ΔG/F had no effect in wild-type cells (Figure 4B), indicating that the negative effects we observed for Sis1ΔG/F are not common to all Hsp40's lacking their G/F regions.

The G/F regions of Ydj1 and Sis1 are similar, but the alignment of these sequences indicates that the larger G/F region of Sis1 has two unique segments, amino acids 67–78 and 101–113 (Figure 4A) (LOPEZ *et al.* 2003). We tested whether overexpression of Sis1Δ67-78, Sis1Δ101-113, or Sis1Δ86-96, which lacks 11 amino acids of the conserved portion of the G/F region, causes growth inhibition. Fourfold overexpression of Sis1Δ67-78 or Sis1Δ86-96 did not inhibit the growth of wild-type cells. However, twofold overexpression of Sis1Δ101-113 in wild-type cells resulted in decreased growth regardless of the temperature at which they were tested (Figure 4C). These results are consistent with the idea that the sequences responsible for the negative effects are those important for Sis1's unique functions such as prion maintenance, as discussed below.

Disruption of interaction with Hsp70 obviates the negative effects of Sis1ΔG/F: Our biochemical results indi-

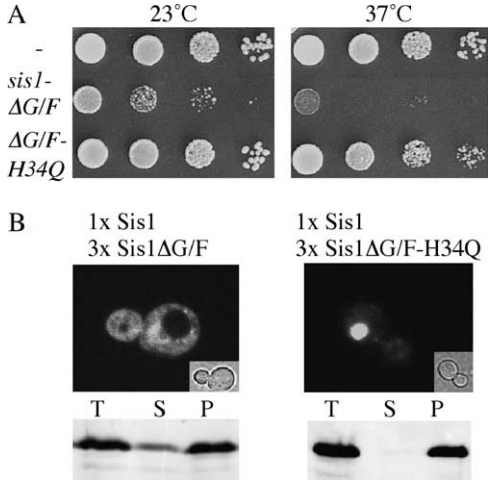

FIGURE 5.—Amino acid substitutions in the J-domain obviate the dominant *in vivo* effects of Sis1ΔG/F on [*RNQ*$^+$] maintenance. Wild-type cells were transformed with plasmids overexpressing either *sis1ΔG/F* (1× Sis1 + 3× Sis1ΔG/F) or *sis1ΔG/F-H34Q* (1× Sis1 + 3× Sis1ΔG/F-H34Q) (A) Tenfold serial dilutions of cells were spotted onto selective medium and incubated for 2 days. (B) Analysis of Rnq1. (Top) Fluorescence microscopy of transformants expressing Rnq1-GFP. (Bottom) Centrifugation analysis of Rnq1. Cell lysate (T), supernatant (S), and pellet (P) fractions were immunoblotted with Rnq1-specific antibodies.

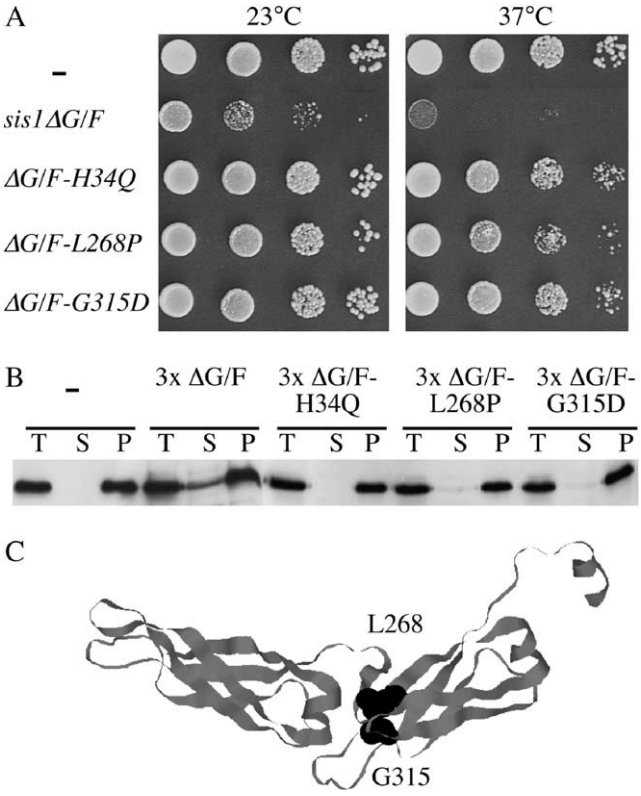

FIGURE 6.—Alterations in the C terminus of Sis1 disrupt the dominant *in vivo* effects of Sis1ΔG/F on growth and [*RNQ*$^+$] maintenance. (A) Serial dilutions of wild-type cells carrying a control vector or a plasmid carrying *sis1ΔG/F, sis1ΔG/F-H34Q, sis1ΔG/F-L268P*, or *sis1ΔG/F-G315D* were grown for 2 days on selective media at 23° or 37°. (B) Centrifugation analysis of Rnq1 using lysates from strains overexpressing *sis1ΔG/F, sis1ΔG/F-H34Q, sis1ΔG/F-L268P*, or *sis1ΔG/F-G315D*. Cell lysate (T), supernatant (S), and pellet (P) fractions were immunoblotted with Rnq1-specific antibodies. (C). Structure of the C-terminal domain of Sis1 (SHA *et al.* 2000) with location of G315 and L268 indicated. Image generated from PDB file 1C3G using Rasmol.

cated that Sis1ΔG/F is not defective in its interaction with substrate proteins or ability to stimulate the ATPase activity of Ssa1, but cannot cooperate in the refolding of denatured luciferase. Because of the dominant effects that we observed, we proceeded to test the hypothesis that an altered interaction, rather than a lack of interaction, of Sis1ΔG/F with Ssa1, is the cause of the inhibitory effect produced by its overexpression. Since the J-domain of Hsp40's interacts with the ATPase domain of Hsp70's, we asked whether a single amino acid alteration in the conserved HPD motif of the J-domain known to disrupt Sis1 function, H34Q (YAN and CRAIG 1999), modulated the dominant effects of Sis1ΔG/F. We found that overexpression of Sis1ΔG/F-H34Q did not inhibit the growth of wild-type cells (Figure 5A).

We also tested whether the H34Q alteration affected the ability of Sis1ΔG/F to disrupt the propagation of the prion. While cells overexpressing Sis1ΔG/F showed dispersed Rnq1-GFP fluorescence, cells overexpressing Sis1ΔG/F-H34Q maintained a single focus of fluorescence (Figure 5B, top). In addition, all the Rnq1 was found in the pellet fraction after high-speed centrifugation of lysates from cells overexpressing Sis1ΔG/F-H34Q (Figure 5B, bottom). These results provide evidence that although Sis1ΔG/F can stimulate the ATPase activity of Ssa1, the interaction between Sis1ΔG/F and Ssa1 is altered and this leads to subsequent loss of [*RNQ*$^+$].

Alterations in the C-terminal domain of Sis1 obviate the toxic effects of Sis1ΔG/F: Since the H34Q alteration obviated Sis1ΔG/F's ability to inhibit growth of wild-type cells, we used error-prone PCR to randomly mutagenize *sis1ΔG/F* and selected for mutations that relieved the growth inhibition (Figure 6A). Two mutants that produce normal levels of protein (data not shown) were selected and analyzed further. Both mutations cause single amino acid alterations in domain II of the C-terminal region (L268P and G315D). Both L268 and G315 lie near the interface with domain I (Figure 6C).

To determine whether the C-terminal mutations that we isolated affect prion maintenance, the aggregation of Rnq1 in cells overexpressing Sis1ΔG/F-L268P or Sis1ΔG/F-G315D was examined. As in the case of cells overexpressing Sis1ΔG/F-H34Q, all of Rnq1 was found in the pellet after high-speed centrifugation of extracts (Figure 6B). In addition, cells overexpressing these proteins maintained a single focus of Rnq1-GFP fluorescence, similar to Sis1ΔG/F-H34Q (data not shown).

Alterations in the C terminus of Sis1 disrupt interaction with the 10-kD domain of Ssa1: Because the C-terminal alterations had the same effects *in vivo* as the J-domain alteration known to affect interactions with Hsp70, we hypothesized that the L268P and G315D alterations affect the interaction of Sis1 with Ssa1. We therefore proceeded to investigate the interaction between the C terminus of Sis1 and Ssa1. Recently, an interaction between the entire C-terminal region of Sis1 (residues 170–352) containing domains I and II, as well as the dimerization domain, and the 10-kD C terminus of Ssa1 was described (Qian *et al.* 2002). While the region of Ssa1 interacting with Sis1 was localized to the most C-terminal 15 residues, the C-terminal segment of the Sis1 involved is not known.

We adapted the previously described ELISA assay to evaluate the interaction between the C terminus of Sis1 and the 10-kD C-terminal domain of Ssa1. The purified 10-kD fragment was bound to the wells of a microtiter plate and the amounts of wild-type or mutant Sis1 proteins retained on the wells after extensive washes were determined using Sis1-specific antibodies. A deletion of the last four C-terminal residues of the 10-kD domain (ΔEEVD), previously shown to be required for Ssa1's interaction with the C terminus of Sis1 (Demand *et al.* 1998; Qian *et al.* 2002), was used as a control. Full-length Sis1 (Figure 7A), as well as the isolated C-terminal region (data not shown), had significant binding to the 10-kD region, while minimal interaction was observed for the control protein, BSA or the 10-kD region containing the EEVD deletion. Full-length Sis1 containing either the L268P or the G315D alteration displayed markedly reduced binding to Ssa1 (Figure 7B), suggesting a role of these residues in the interaction between Sis1 and the 10-kD region of Ssa1. These results provide additional evidence that altered interaction of Sis1ΔG/F with Ssa1 leads to the dominant effects of the mutant protein on [*RNQ*$^+$] maintenance and cell growth.

***In vivo* consequences of defective interaction between the C terminus of Sis1 and the 10-kD domain of Ssa1:** To test the effect of amino acid alterations in the C terminus of Sis1 that alter its interaction with the 10-kD domain of Ssa1, we examined the phenotype of *sis1-L268P* and *-G315D* in the absence of wild-type Sis1. Serial dilutions of strains expressing only Sis1-L268P or Sis1-G315D were tested for growth at 23°, 30°, and 37°. No growth defects compared to cells expressing wild-type Sis1 were observed (Figure 8A). All three strains produced the same level of Sis1 protein as judged by immunoblot analysis (data not shown). [*RNQ*$^+$] phenotypes of *sis1-L268P* and *sis1-G315D* were also compared with those of wild type and *sis1ΔG/F*. Cells expressing Sis1-L268P or Sis1-G315D showed an intermediate [*RNQ*$^+$] phenotype. Rnq1-GFP fluorescence was visible as a pattern of multiple small foci in virtually all cells

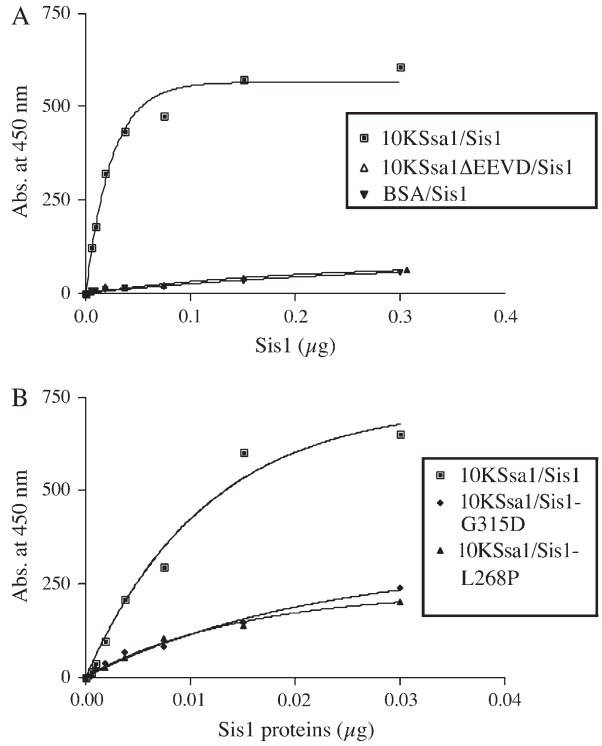

Figure 7.—Alterations in the C terminus of Sis1 disrupt its interaction with the 10-kD region of Ssa1. (A) Binding of Sis1 to the purified 10-kD fragment of Ssa1 (10KSsa1), 10KSsa1 lacking the C-terminal four amino acids (10KSsa1ΔEEVD), or BSA as a control. A total of 7 pmol of 10-kD fragment (or BSA) was immobilized in wells of a microtiter plate and incubated with serial dilutions of purified Sis1. Sis1 retained in the wells after washing was detected by immunoblot analysis using antibody specific for Sis1. (B) Binding of Sis1, Sis1-L268P, or G315D to the 10KSsa1. A total of 7 pmol of purified 10KSsa1 was immobilized in wells of a microtiter plate and serial dilutions of Sis1, Sis1-G315D, or L268P were added. Sis1 retained in the wells was detected by antibody specific for Sis1.

(Figure 8B). In addition, a portion of Rnq1 was present in the soluble fraction (Figure 8B). Thus, alteration of these residues affects the prion state of Rnq1, although not as dramatically as the absence of the G/F region.

DISCUSSION

Bipartite interaction between Sis1 and Hsp70. The results presented here indicate that a bipartite interaction between Sis1 and Ssa1 occurs *in vivo*. It has been appreciated for some time that Hsp40's and Hsp70's interact, as binding of the J-domain of Hsp40 to the Hsp70 ATPase domain is required for stimulation of ATPase activity (Bukau and Horwich 1998; Laufen *et al.* 1999). A second site of interaction between the 10-kD domain of Ssa1 and the C-terminal 181 amino acids of Sis1 was recently described (Qian *et al.* 2002). The suppressor analysis described above uncovered two alterations in Sis1 that disrupted this interaction, L268P

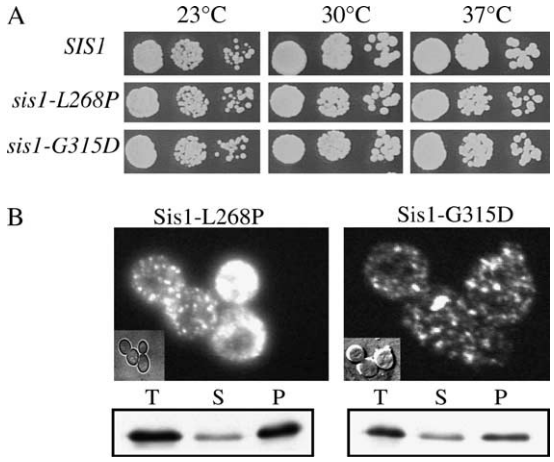

FIGURE 8.—Phenotypes of strains expressing *sis1-L268P* and *sis1-G315D*. *sis1::LEU2/*YCp50-*SIS1* was transformed with a plasmid expressing *SIS1*, *sis1-L268P*, or *sis1-G315D*. Transformants having lost the YCp50-*SIS1* plasmid were selected on plates containing 5-FOA. (A) Serial dilutions of cells expressing the indicated genes were grown for 2 days on rich media at the indicated temperatures. (B) Analysis of Rnq1. (Top) Fluorescence microscopy of indicated transformants expressing Rnq1-GFP. (Bottom) Centrifugation analysis of Rnq1 using lysates from *sis1* strain expressing wild-type *SIS1*, *sis1ΔG/F*, *sis1-L268P*, or *sis1-G315D*. Cell lysate (T), supernatant (S), and pellet (P) fractions were immunoblotted with Rnq1-specific antibodies.

and G315D. Both mutated residues are in domain II of the C-terminal region of Sis1, very close to the interface with domain I. Sequence comparison indicated that residue L268 is not strictly conserved, although most Sis1 homologs have a hydrophobic residue at this position. In contrast, residue G315 is highly conserved, being found in all Hsp40's examined. Alterations of highly conserved residues in the α-helix of domain I that faces domain II (L190A and E191A) also relieve the growth inhibition caused by Sis1ΔG/F (data not shown), suggesting that these alterations also disrupt Sis1-Ssa1 interaction. On the basis of the available structural information (SHA *et al.* 2000; LI *et al.* 2003) and functional studies of the putative peptide-binding region (JOHNSON and CRAIG 2001; LEE *et al.* 2002), these residues lie neither within the substrate-binding surface nor within the dimerization domain of Sis1. Thus the interface between the two domains may be the site of interaction with the 10-kD domain of Ssa1.

Bipartite interactions between an Hsp70 such as Ssa1 and an Hsp40 such as Sis1 can easily be envisioned as an important part of a mechanism by which substrates are efficiently transferred from an Hsp40 to an Hsp70. The interaction between more than one site on the proteins, the J-domain with the ATPase domain of Ssa and the C terminus with the 10 kD of Ssa, could serve to correctly align the two proteins, thus assisting in the transfer step. With Sis1 as a dimer, an interaction between the C termini of the two proteins can be modeled such that Sis1 "straddles" the peptide-binding domain of Ssa in a position to readily achieve substrate delivery (LANDRY 2003).

Whether such a bipartate interaction occurs with other Hsp40's is unresolved. Using the same assay used to analyze Sis1 and Ssa1, no interaction between Ydj1 and the 10-kD domain of Ssa1 was detected (data not shown). Either the C-terminal interaction with the 10-kD domain may be restricted to Hsp40's of the Sis1 class or such an interaction may occur with other Hsp40's, but with weaker contacts, and thus is not detectable under the conditions used. In fact there is evidence that interactions occur between the Ydj1/DnaJ type of Hsp40's and the peptide-binding domain of Hsp70's (GÄSSLER *et al.* 1998; SUH *et al.* 1998; LINKE *et al.* 2003), although the nature of this interaction has not been elucidated.

The question of the functional importance of the glycine-rich and the C-terminal regions of Sis1 presents apparent conundrums. On the one hand, the data discussed above provide evidence for *in vivo* interaction between the C terminus of Sis1 and the C terminus of Hsp70 and indicates that disruption of this interaction has effects *in vivo* on prion maintenance. On the other hand, a Sis1 fragment containing only the J-domain and G/F region (Sis1-121) is able to function *in vivo* (YAN and CRAIG 1999; SONDHEIMER *et al.* 2001). Sis1-121 is sufficient to maintain both cell viability and the prion form of Rnq1, albeit in the visibly less aggregated form, similar to that observed for Sis1-L268P and -G315D. In addition, single amino acid alterations in the G/F region of Sis1-121 that drastically affect both prion maintenance and essential cellular functions have been isolated (LOPEZ *et al.* 2003). These results indicate that the G/F region can perform specific important functions. Thus, past suggestions that the glycine-rich regions of Hsp40's serve only as flexible linkers between important domains is not the case, at least for Sis1.

We favor the idea that different regions of Sis1 function together to efficiently transfer substrates to Hsp70. Efficient "targeting" is dependent on specific sequences in the glycine-rich region, as well as on those in the carboxy-terminal regions that interact with the 10-kD regulatory region of Ssa1. According to this model, coordinated interaction of these regions of Sis1 with Ssa1 is required to facilitate transfer of substrates bound to Sis1 to the Ssa1 peptide-binding domain. Maintenance of [*RNQ*$^+$] seems particularly dependent on robust Sis1 function, as both deletion of the G/F region, and the single amino acid substitutions Sis1-L268P and Sis1-G315D, have more severe effects on prion maintenance than on cell growth.

Why does overexpression of Sis1ΔG/F have dominant deleterious effects? One possibility is that deletion of the G/F region of Sis1 changes the spacing between the J-domain and the C-terminal regions. One could

imagine that the spacing between the two sites of interaction between Ssa's ATPase and C-terminal domains is critical and alteration of this spacing interferes with productive transfer of substrate protein. However, this idea is not favored by the analysis of other deletions, particularly as contrasting results were observed with the smaller deletions, Sis1Δ67-78 or Sis1Δ101-113. Only deletion of residues 101–113 caused a dominant deleterious defect similar to deletion of the entire G/F region. Interestingly, residues within this region were found to be critical for Sis1-121's ability to rescue growth of a Sis1 deletion strain and to maintain [*RNQ*$^+$] (Lopez *et al.* 2003). Thus we favor the idea that mutant Sis1 proteins have lost some regulatory functions, resulting in deleterious effects. Perhaps a complex of Sis1ΔG/F:substrate polypeptide:Ssa1 is stabilized, compared to the very transient interaction of the wild-type Sis1:substrate complex with Ssa1. Such stabilization would be expected to be deleterious for the cell. This idea would be consistent with the fact that mutations that cause destabilization of the Sis1:Ssa1 interaction are suppressors of the dominant effects of *sis1ΔG/F*.

A role for the G/F region in "targeting" of substrates is likely not unique for Sis1, as an *E. coli* mutant DnaJ lacking the G/F region was shown to be defective in enhancing binding of the Hsp70 DnaK to the native substrate σ^{32} (Wall *et al.* 1995), although the basis of this defect was not pursued. How a G/F region functions in such "targeting" is still a matter of conjecture, but two alternatives can be envisioned. First, the G/F region may directly affect the spatial orientation of J-domain residues, with different G/F regions subtly altering the overall J-domain conformation, and thus its interaction with Hsp70 in such a way that substrate specificity is altered. Evidence does suggest that the presence of the G/F region alters the structure of the J-domain of DnaJ (Huang *et al.* 1999), but the importance of this alteration is unknown. Alternatively, the G/F region could directly interact with Hsp70, affecting its structure. Tests of these models are in progress. Either way, since Sis1 and Ssa1 have roles in maintaining [*RNQ*$^+$] (Lopez *et al.* 2003), interaction with a specific Hsp40 provides a mechanism to specialize Hsp70 function.

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