

Specificity of Class II Hsp40 Sis1 in Maintenance of Yeast Prion [RNQ⁺]

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Sis1 and Ydj1, functionally distinct heat shock protein (Hsp)40 molecular chaperones of the yeast cytosol, are homologs of Hdj1 and Hdj2 of mammalian cells, respectively. Sis1 is necessary for propagation of the *Saccharomyces cerevisiae* prion [RNQ⁺]; Ydj1 is not. The ability to function in [RNQ⁺] maintenance has been conserved, because Hdj1 can function to maintain Rnq1 in an aggregated form in place of Sis1, but Hdj2 cannot. An extended glycine-rich region of Sis1, composed of a region rich in phenylalanine residues (G/F) and another rich in methionine residues (G/M), is critical for prion maintenance. Single amino acid alterations in a short stretch of amino acids of the G/F region of Sis1 that are absent in the otherwise highly conserved G/F region of Ydj1 cause defects in prion maintenance. However, there is some functional redundancy within the glycine-rich regions of Sis1, because a deletion of the adjacent glycine/methionine (G/M) region was somewhat defective in propagation of [RNQ⁺] as well. These results are consistent with a model in which the glycine-rich regions of Hsp40s contain specific determinants of function manifested through interaction with Hsp70s.

INTRODUCTION

Several prion proteins have been identified in *Saccharomyces cerevisiae*. Such proteins have the unusual ability to exist in distinct and heritable states (Wickner and Masison, 1996; Lindquist, 1997). The stability of these states allows for the faithful transmission of the prions during cell division. Genomic searches for Gln/Asn-rich domains, a domain critical for prion propagation, led to the identification of another prion protein, Rnq1 (Sondheimer and Lindquist, 2000). Like other prions, Rnq1 exists in two heritable states, a soluble form, [rnq⁻], and an aggregated form, [RNQ⁺], also known as the epigenetic element [PIN⁺] (Derkatch *et al.*, 2001).

Molecular chaperones, known to function in a variety of cellular processes involving changes in protein conformation (Hartl, 1996; Bukau and Horwich, 1998; Craig *et al.*, 1999), have recently been implicated in the maintenance of the prion form of proteins. Heat shock protein (Hsp)104 is required for the maintenance of the yeast prion states [PSI⁺] and [RNQ⁺] (Chernoff *et al.*, 1995; Patino *et al.*, 1996; Sondheimer and Lindquist, 2000). The efficient propagation of

[PSI⁺] also requires the Hsp70 Ssa1 (Jung *et al.*, 2000). Moreover, it was demonstrated that the function of the Hsp40 Sis1 was required for [RNQ⁺] propagation (Sondheimer *et al.*, 2001). However, little is understood about the mechanism by which one or another molecular chaperone affects prion propagation.

Hsp40s, J-type proteins that function as molecular chaperones with Hsp70s (Kelley, 1998), assist Hsp70s function in two ways: 1) stimulating the intrinsic ATPase activity of Hsp70, thus promoting a relative stable complex between Hsp70 and unfolded polypeptides; and 2) in various cases Hsp40s can bind unfolded polypeptides themselves and transfer them to Hsp70s (Hartl, 1996; Bukau and Horwich, 1998). Working together, Hsp40s and Hsp70s facilitate the refolding of denatured proteins in vivo and in vitro (Kim *et al.*, 1998; Lu and Cyr, 1998a,b; Liu *et al.*, 2001).

Hsp40s have been divided into two classes (Cheetham and Caplan, 1998). Members of both classes have a signature J domain of ~70 amino acids that is critical for the interaction with Hsp70s. A glycine-rich region follows the J domain of both classes, which are often rich in phenylalanine residues and vary in length. For instance, the glycine-rich region of Sis1, the focus of this work, has an extended glycine-rich region with one segment rich in phenylalanine residues (G/F) and a second rich in methionine residues (G/M). Adjacent to the glycine-rich region, class I Hsp40s have a cysteine-rich region, followed by an unconserved C terminus, whereas class II Hsp40s lack a cysteine-rich region, but do have an unconserved C-terminal region. The uncon-

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served C-terminal region of both classes contains the unfolded polypeptide binding region (Banecki *et al.*, 1996; Szabo *et al.*, 1996; Sha *et al.*, 2000).

Two Hsp40s of the yeast cytosol are well characterized: class I Ydj1 and class II Sis1. In vivo analyses indicate that Ydj1 is involved in the translocation of proteins from the cytosol into organelles (Becker *et al.*, 1996). Sis1 has been linked to the initiation of protein synthesis (Zhong and Arndt, 1993). However, it is likely that both of these Hsp40s play multiple roles in the cytosol. Both can stimulate the ATPase of the Ssa class of Hsp70s, and function with Ssa1 to facilitate the refolding of denatured proteins (Lu and Cyr, 1998b), suggesting that they function with the same Hsp70 in vivo.

The functional relationship between these two Hsp40s in vivo is complex. *SIS1* is an essential gene (Luke *et al.*, 1991); *YDJ1* is not essential, but cells lacking Ydj1 grow very poorly (Caplan and Douglas, 1991). Overexpression of Sis1 can suppress the slow growth phenotype of a *ydj1* strain (Caplan and Douglas, 1991), but overexpression of Ydj1 cannot rescue a *sis1* strain (Luke *et al.*, 1991). Therefore, Sis1 can carry out Ydj1 functions, but Sis1 performs an essential function in vivo that Ydj1 cannot.

Genetic analysis has implicated the G/F segment as the region defining Sis1-specific function. A Sis1 truncation having only the J domain and the G/F region is able to rescue growth of a Δ *sis1* strain (Yan and Craig, 1999). In this 121 amino acid fragment, the critical region for Sis1-specific function is the G/F region, as a chimeric gene encoding the J domain of Ydj1 and the G/F region of Sis1 allows growth of Δ *sis1* cells. The importance of the G/F region is also indicated by the fact that a Sis1 protein lacking only the G/F region is unable to maintain the prion form of Rnq1, although it still allows growth (Sondheimer *et al.*, 2001). Because of the importance of the G/F region in Sis1 function, we pursued an analysis of its specificity.

MATERIALS AND METHODS

Yeast Strains and Growth Methods

The following W303 isogenic strains were used in this study: wild-type PJ51-3A (*MAT a trp1-1 ura3-1 leu2-3112 his3-11,15 ade2-1 can1-100 GAL2+ met2- Δ 1 lys2- Δ 2*) (James and Craig, unpublished data); WY26 (*MAT α lys2- Δ 2 Δ sis1::LEU2*) (Yan and Craig, 1999); JJ160 (*MAT a ydj1::HIS3*) (Johnson and Craig, 2000). 74D-694 Δ *sis1* [*RPS*⁺] (*MAT a ade1-14 trp his leu ura sup35::RMC Δ sis1::KAN*) (Sondheimer *et al.*, 2001) was used for analysis of the synthetic prion protein RMC. Yeast cells were grown on rich medium (YPD) or minimal medium (SD) where specified (Sherman *et al.*, 1986).

For testing the ability of *sis1* mutants to support in vivo functions, "plasmid shuffling" (Sikorski and Boeke, 1991) was used as described elsewhere (Yan and Craig, 1999) by using WY26 cells. The growth of *sis1* mutants was evaluated on YPD and SD media with 5-fluororotic acid (5-FOA) after 3 d of incubation at 30°C. Plasmid shuffling was also used to test the ability of full-length *sis1gff* mutants to support [*RPS*⁺] maintenance in 74-D694 Δ *sis1* [*RPS*⁺] cells. Growth was evaluated on YPD after 3 d of incubation at 30° and on SD media lacking adenine after 5 d at 30°C.

Plasmids

Plasmids used in this study: pYW65 (pRS314-*SIS1*), pYW116 (pRS314-*sis1 Δ gff*), pYW66 (pRS314-*sis1-171*), pYW62 (pRS314-*sis1-121*), pYW70 (pRS314-*YS-121*), p316-*RNQ1-GFP* have been de-

scribed elsewhere (Yan and Craig, 1999; Sondheimer and Lindquist, 2000). p314-*sis1 Δ g/m* was created by engineering an internal deletion on pYW65 that removes sequences encoding from amino acid 121–170. *psis1-121-N108I* and *psis1-121-D110G* were isolated from a genetic screen by using a plasmid library originated from polymerase chain reaction (PCR) fragments with random mutations cloned onto pYW62. *psis1-121-D110N* was a single mutation created on pYW62 based on an isolated full-length *sis1* mutant (*SIS1-D110N*). *psis1 Δ g/m-N108I* and *psis1 Δ g/m-D110G* resulted from generating a single mutation in p314-*sis1 Δ g/m*. *psis1-N108I* and *psis1-D110G* were obtained by introducing the 3'-end sequence of *SIS1* in *psis1-121-N108I* and *psis1-121-D110G*. p314-*sis1-121 Δ 67-78* and p314-*sis1-121 Δ 101-113* were internal deletions generated on pYW62 by PCR reactions. Construction of p314-*sis1- Δ g/m Δ 67-78* and p314-*sis1- Δ g/m Δ 101-113* were done by cloning *sis1-121* from p314-*sis1-121 Δ 67-78* or p314-*sis1-121 Δ 101-113* into *psis1 Δ g/m*. p314-*sis1 Δ 67-78* and p314-*sis1 Δ 101-113* were made as described above for *psis1-N108I* and *psis1-D110G*.

Human *HDJ1* was cloned from pET3a-hHsp40 (Minami *et al.*, 1996) into pUC21 and subcloned into a pRS424 *GPD* driven expression vector (424*GPD-HDJ1*). To create *HDJ1* mutant clones, a stop codon was engineered by PCR at amino acid position 147 amino acids (*HDJ1-147*) followed by its 3'-end untranslated region. Resulting PCR products were cloned into the pRS424 *GPD* vector. The *Drosophila melanogaster* Hsp40 *DROJ1* was cloned from pYX232*DroJ1* (Marchler and Wu, unpublished data) to pUC21 and subcloned to the pRS424 *GPD* vector. Construction of *droj1* mutant clones was achieved by generating a stop codon by using PCR at amino acid position 143 (*DROJ1-143*) followed by its 3'-untranslated region. PCR fragments were cloned into pRS424 *GPD*-driven expression vector. *HDJ2* was amplified by PCR from a plasmid (Toft, unpublished data) and cloned into a pRS424 *GPD* promoter vector.

A plasmid library of *sis1-121gff* containing random mutations in the coding sequence of the G/F region was created from the plasmid pYW62 by using error prone PCR (Yan and Craig, 1999). PCR-amplified DNA was cloned into pYW62 to replace the wild-type sequence of the G/F region. About 9000 independent *Escherichia coli* transformants were obtained with 1% of the population containing clones lacking inserts.

A second mutant library was derived from the *sis1-121gff* mutant library to identify the sequences within Sis1's G/F region required to support prion maintenance. *Bam*HI-*Not*I DNA fragments were subcloned into the same sites of YCplac-*SIS1*, replacing the indicated sequences in this wild-type gene. Ligations were transformed into *E. coli* and resulted in ~26,000 independent transformants with 20% of the population lacking inserts.

Protein Analysis

Immunoprecipitations and aggregation assays were carried out as described elsewhere (Sondheimer *et al.*, 2001). In aggregation assays, cell lysates were fractionated by differential centrifugation at 280,000 × g. Protein quantifications with the wild-type strain PJ51-3A was performed as described elsewhere (Pfund *et al.*, 1998) by using purified Sis1 and Rnq1 as standards.

Purified Rnq1 and its specific polyclonal antibodies were kindly provided by Neal Sondheimer and S. Lindquist (Sondheimer and Lindquist, 2000). In addition, Rnq1 antibodies were generated using as an immunogen the first 185 amino acids of Rnq1 fused to glutathione S-transferase (GST). This GST-Rnq1-185 fusion was expressed in *E. coli* and purified using glutathione beads, and then injected into rabbits to raise polyclonal antibodies. Purified Sis1 was obtained from WY26 cells expressing histidine-tagged Sis1 from the plasmid p424-*GPD-HisB-SIS1* by using Nickel chelate affinity chromatography (His-Bind Resin from Novagen, Madison, WI).

Fluorescence Microscopy

For fluorescence microscopy, cells were transformed with a copy of *RNQ1-GFP* that is regulated by the *CUP1* promoter (Sondheimer

and Lindquist, 2000). Transformants in mid-log phase were induced by the addition of 50 μM CuSO_4 and then incubated for 4 h at 30°C. After induction, cells were mixed with an equivalent amount of 1% SeaPlaque agarose (FMC Products, Rockland, ME) and visualized in a fluorescence microscope Axioplan 2 (Carl Zeiss, Thornwood, NY). Digital images were captured using the QED software (QED Imaging, Pittsburgh, PA).

RESULTS

Sis1 and Rnq1 Are Present in Equimolar Amounts in Immunoprecipitates of Prion [RNQ⁺]

Sis1 can be coimmunoprecipitated with the prion, but not the soluble, form of Rnq1 (Sondheimer *et al.*, 2001). To further characterize the Sis1:Rnq1 interaction found in [RNQ⁺] cells, we assessed the amounts of Sis1 and Rnq1 in these complexes by using Sis1-specific antibodies in immunoprecipitation assays. Nearly all of the Rnq1 in [RNQ⁺] lysates was associated with Sis1 (Figure 1A). However, Sis1 was determined to be ~65 times more abundant in cell lysates than Rnq1 (Figure 1B). Therefore, the majority of Sis1 is available for other interactions, including those essential for cell viability.

To determine the amount of Sis1 relative to Rnq1 in the Sis1:Rnq1 complexes, we carried out immunoprecipitation assays by using antibodies against Rnq1. Only ~2% of the total amount of Sis1 was in association with Rnq1 (Figure 1C). Rnq1 and Sis1 are therefore found in approximately equimolar amounts in these complexes, consistent with an important role of Sis1 in prion maintenance.

Ydj1 Can Associate with the prion [RNQ⁺], but Is Dispensable for Its Propagation

Another Hsp40, Ydj1, which is ~10 times more abundant than Sis1 (Yan and Craig, 1999), is present in the yeast cytosol. To address whether Ydj1 is also involved in prion maintenance, immunoprecipitates isolated using Rnq1-specific antibodies were tested for the presence of Ydj1 by immunoblot analysis by using Ydj1-specific antibodies. Low amounts of Ydj1 were consistently coimmunoprecipitated with the prion [RNQ⁺] (Figure 2A).

To determine the biological significance of the Ydj1 association with [RNQ⁺], *ydj1* cells were evaluated for the stability of [RNQ⁺]. The distribution of Rnq1 in living cells after transient overexpression of an Rnq1-green fluorescent protein fusion (Rnq1-GFP) was monitored. As previously shown, the fluorescence showed as a single focus in the majority of wild-type [RNQ⁺] cells (Sondheimer and Lindquist, 2000), whereas the remaining cells had multiple small foci. As typically seen in [*rnq*⁻] cells, the Rnq1-GFP fluorescence was diffuse in cells carrying Sis1 lacking the G/F region (Sis1 $\Delta\text{G}/\text{F}$) (Figure 2B; Sondheimer *et al.*, 2001). Fluorescence microscopy of a *ydj1* null mutant expressing RNQ1-GFP was indistinguishable from a [RNQ⁺] wild-type strain (Figure 2B).

This *in vivo* approach was complemented by assaying cell lysates for the presence of the prion by high-speed centrifugation. Rnq1 from wild-type cells was all in the pellet fraction, whereas it was all of Rnq1 was in the supernatant fraction of *sis1* $\Delta\text{g}/\text{f}$ lysates. As in analysis of lysates from a wild-type strain, all of the Rnq1 from the *ydj1* cells was found in the pellet fractions, indicating that the [RNQ⁺]

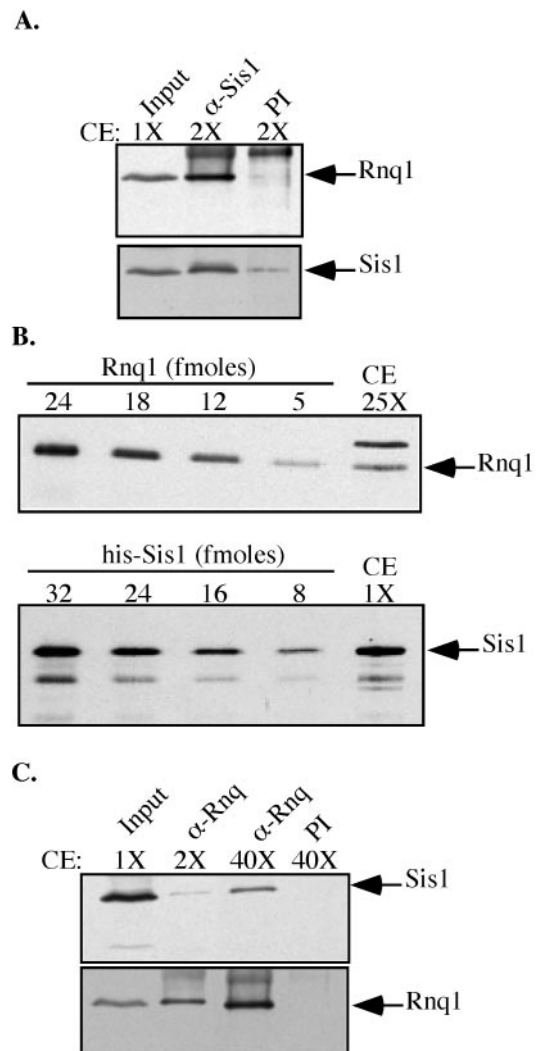


Figure 1. Sis1 coimmunoprecipitates with the prion form of Rnq1 in equimolar amounts. (A) Immunoprecipitation assays performed with lysates from an [RNQ⁺] W303 wild-type strain by using Sis1-specific antibodies. Relative amounts of cell extract (CE) loaded directly as input or used in immunoprecipitations are indicated as (1×) or two times (2×) with Sis1-specific antibodies (α -Sis1) or preimmune sera (PI). Relative amounts of proteins were determined by immunoblot analysis by using specific antibodies against Sis1 and Rnq1, followed by densitometry measurements. The efficiency of immunoprecipitation of Sis1 by α -Sis1 was ~50%. (B) To determine the relative abundance of Rnq1 and Sis1 in extracts of [RNQ⁺] cells, dilution series of purified Rnq1, histidine-tagged Sis1 (his-Sis1) and CE were separated by SDS-PAGE. Numbers represent the femtomoles of purified proteins in each lane. Various dilutions of cell extract were loaded on gels but only those dilutions used for quantification are shown. The total amount of protein loaded for cell extract (CE1X) was 0.5 μg ; in CE25X 12.5 μg . Proteins were resolved by electrophoresis and analyzed by immunoblot analysis by using antibodies against Rnq1 or Sis1. The amount of Rnq1 in 25X cell extract sample was determined to be 12 fmol, whereas 31 fmol Sis1 was in 1× cell extract sample. (C) Immunoprecipitation assays with Rnq1 specific antibodies (α -Rnq1) as described above. When 2× cell lysate was used the efficiency of immunoprecipitation by α -Rnq1 was ~50%.

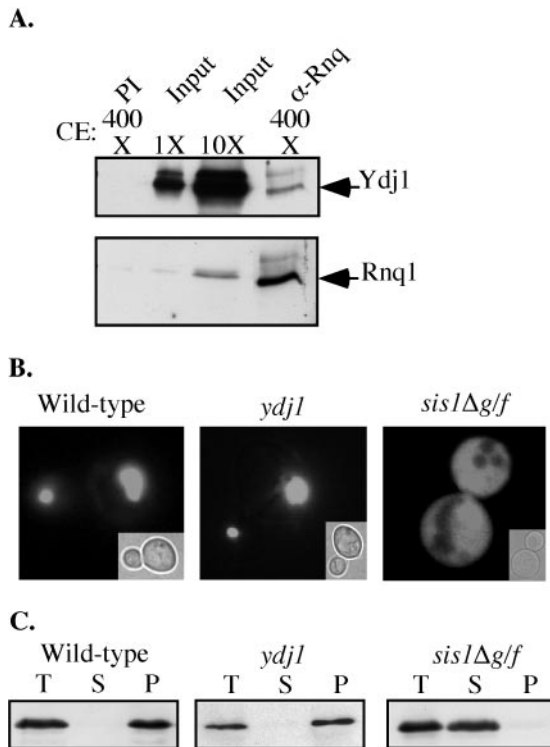


Figure 2. Ydj1 is dispensable for maintenance of the prion [RNQ⁺]. (A) Coimmunoprecipitation of Rnq1 and Ydj1. Immunoprecipitation assays performed with lysates from an [RNQ⁺] W303 wild-type strain by using Rnq1-specific antibodies (α -Rnq1) or preimmune serum (PI) as a control. We loaded $\sim 2.5 \mu\text{g}$ (1X) and $25 \mu\text{g}$ (10X) of total protein in lysate on a SDS-polyacrylamide gel together with the total immunoprecipitated material from $400 \mu\text{l}$ (400X) of lysate containing $\sim 1 \text{ mg}$ of total protein. Samples were subjected to electrophoresis followed by immunoblot analysis by using specific antibodies against Ydj1 and Rnq1. (B) Fluorescence microscopy of Rnq1-GFP in wild-type and *ydj1* cells. The aggregation state of Rnq1-GFP was assessed by fluorescence microscopy after Rnq1-GFP expression was induced with $50 \mu\text{M}$ CuSO_4 for 4 h. Fluorescent images are representative of $>60\%$ of cells in the population. Insets, corresponding bright field images. (C) Aggregation assays for Rnq1 were carried out by fractionating crude lysates from a wild-type or *ydj1* strain by using high-speed centrifugation. Equivalent aliquots of total (T) lysate, supernatant (S), and pellet (P) fractions were separated by SDS-PAGE, followed by immunoblot analysis by using Rnq1-specific antibodies.

prion is maintained in the absence of Ydj1 (Figure 2C). Therefore, Ydj1 is dispensable for the propagation of the prion [RNQ⁺], whereas Sis1 plays a specific role in this process.

In Vivo Sis1 Functions Are Complemented by the Human and *D. melanogaster* Sis1 Homologs

Hsp40s related to Sis1 and Ydj1 have been conserved during evolution. For example, Hdj1 and Hdj2 are human homologs of Sis1 and Ydj1, respectively. In addition, it was recently found that the *Drosophila* homolog of Sis1 would rescue the viability of a Δ *sis1* strain (Marchler and Wu, 2001).

To test whether any of these heterologous proteins could function to maintain [RNQ⁺], a Δ *sis1* strain expressing *SIS1* from a *URA3*-based centromeric plasmid was transformed with a *TRP1*-based plasmid containing either *HDJ1*, *HDJ2*, or *DROJ1* driven by the yeast *GPD1* promoter. Growth of selected transformants was evaluated on media containing 5-FOA, which selects for cells having lost the *URA3*-based plasmid (Sikorski and Boeke, 1991). Cells expressing either Hdj1 or Droj1 grew on media containing 5-FOA, but cells expressing Hdj2 did not (Figure 3A), indicating that both these class II Hsp40s can carry out the essential functions of Sis1, but the class I Hdj2 cannot. However, Hdj2 allowed wild-type growth of a *ydj1* strain at 30°C , and thus is functional as a class I Hsp40 in yeast.

Because both Hdj1 and Droj1 were able to maintain the viability of Δ *sis1* cells, we asked whether cells having either of these heterologous proteins were able to maintain Rnq1 in the prion form. All of the Rnq1 protein was in the pellet fraction after sedimentation of lysates of Δ *sis1* cells expressing either Hdj1 and Droj1, indicating that Rnq1 was in the prion form (Figure 3B). In the *in vivo* assay with GFP-Rnq1, the majority of cells showed multiple small foci, with only a few cells showing a single center of fluorescence, most commonly seen in wild-type cells.

This pattern of multiple small foci resembled that observed in cells expressing Sis1-121, which contains only the J domain and G/F region of Sis1. Therefore, we proceeded to further dissect the sequences in Hdj1 and Droj1 sufficient for supporting growth as well as prion maintenance. Truncation mutants of both *HDJ1* and *DROJ1* that encoded only the J domain and glycine-rich region, Hdj1-147 and Droj1-143, were constructed. Both were able to rescue the growth of a Δ *sis1* strain and maintain the Rnq1 prion (Figure 3C; our unpublished data). Thus, the specificity of the Sis1-type (class II) Hsp40 has been conserved in evolution, and residues within the J domain:G/F region are sufficient for both Sis1's essential function and prion maintenance.

G/F Region Carries the Specificity for Prion Maintenance

The results described above demonstrate that the J domain and G/F region of Sis1 homologs from diverse organisms are sufficient to maintain [RNQ⁺]. Previously, the G/F region was shown to be required for Sis1's specific function(s) in maintaining cell viability, because the chimera between the J domain of Ydj1 and the G/F region of Sis1 (YS-121) was sufficient to allow growth of a Δ *sis1* strain (Yan and Craig, 1999). Therefore, using this chimeric gene, we tested whether the Sis1 J domain was critical for prion maintenance. A strain containing YS-121 as the only protein having Sis1 G/F sequences was able to maintain Rnq1 in the prion form as indicated by both centrifugation and fluorescence assays (Figure 3D). Thus, Sis1's J domain is functionally exchangeable with that of Ydj1 but its G/F region contains sequences critical for maintenance of the Rnq1 prion.

A Unique Amino Acid Sequence (aa 101–113) in the *G/F* Region Determines Sis1's Functional Specificity

Sis1, but not Ydj1, is required for maintenance of the Rnq1 prion. Our observations indicate that the functional distinction between Sis1 and Ydj1 in prion maintenance is attrib-

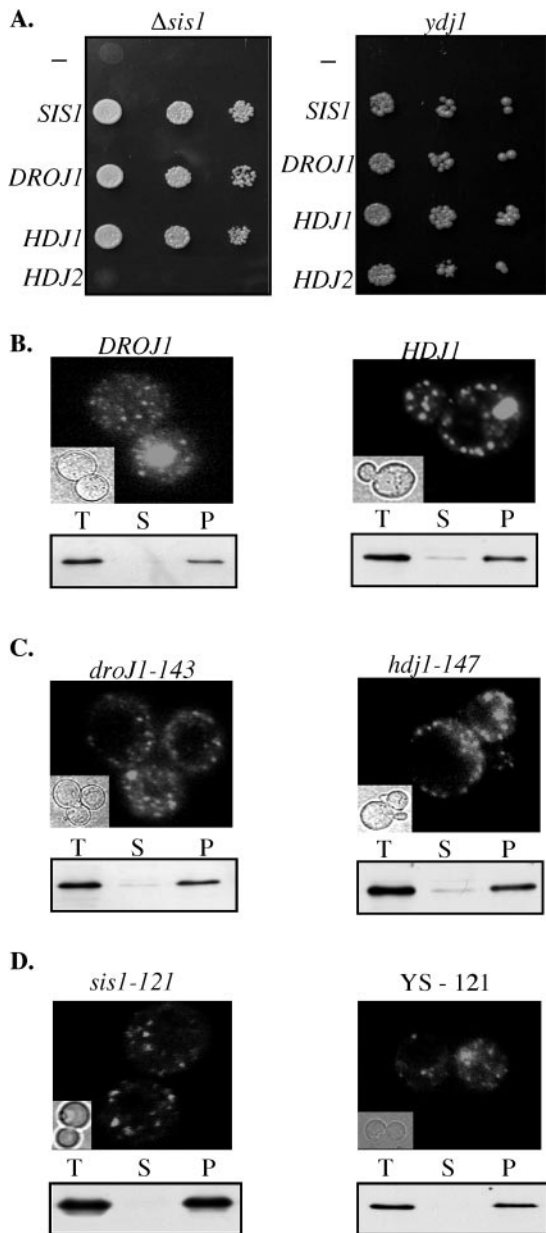


Figure 3. J domain and G/F region of *Drosophila* and human Sis1 homologs can complement Sis1's roles in cell growth and $[RNQ^+]$ maintenance. (A) A 10-fold dilution series of cell suspensions from a $\Delta sis1$ (left) or $ydj1$ (right) strain carrying *SIS1* or *YDJ1*, respectively, on a *URA3*-containing vector and second plasmid having no Hsp40 gene (-), *SIS1*, *Drosophila DROJ1*, human *HDJ1*, or human *HDJ2*. Cells were spotted on media containing 5-FOA and incubated for 3 d. (B–D) Top, fluorescence microscopy of $\Delta sis1$ cells coexpressing an Hsp40 gene and *RNQ1-GFP*. Cells were visualized 4 h after *RNQ1-GFP* induction as described above. Bottom, aggregation assays for the lysates of corresponding strains. After centrifugation, equivalent aliquots of total lysate T, supernatant fraction S, and pellet fraction P were subjected to immunoblot analysis by using *RNQ1*-specific antibody. (B) Full-length *DROJ1* and *HDJ1*. (C) Truncation mutants of *DROJ1* and *HDJ1*, *droJ1-143* and *hdj1-147*, which encompass their corresponding J domain and glycine-rich sequences. (D) *Sis1-121* truncation or a fusion that includes the J domain of *Ydj1* fused to the G/F region of *Sis1* (YS-121).

utable to the G/F region. Thus, we compared the sequences within the G/F region between the two Hsp40s (Figure 4A). The G/F regions have extensive conservation in the glycine-rich segments, as well as in a small segment at the C terminus of this region that is devoid of glycine residues. However, the *Sis1* G/F region is longer than that of *Ydj1* due to two unique stretches of amino acids (aa 67–78 and 101–113).

To determine the importance of these unique segments of the G/F region for function, internal deletion mutants of *sis1-121* that encode proteins lacking one or the other of the unique segments of the *Sis1* G/F region were created. Cells expressing *sis1-121* $\Delta 67-78$ were viable. In contrast, *sis1-121* $\Delta 101-113$ could not support growth (Figure 4B), even though protein levels similar to *sis1-121* were produced (our unpublished data).

Because the N-terminal 121 amino acids of *Sis1* (*Sis1-121*) are sufficient to maintain *RNQ1* in the prion state, as well as support viability, we also tested the state of the *RNQ1* prion in *sis1-121* $\Delta 67-78$ cells. All of *RNQ1* was found in the pellet fractions after differential centrifugation (Figure 4B). Therefore, the unique stretch of amino acids between positions 67 and 78 is not important for either cell viability or prion maintenance.

Single Amino Acid Alterations within the aa 101–113 Segment of the G/F Region Alleviate the Ability of *Sis1-121* to Support Cell Viability

Because the results described above suggest a critical role of the amino acids between positions 101–113 in the G/F region in maintaining the prion $[RNQ^+]$, we wanted to identify amino acids within this region important for this function. In the absence of a phenotype that distinguishes $[RNQ^+]$ from $[rnq^-]$ cells, we used an alternative genetic screen based on the ability of *Sis1-121* to support cell viability (Yan and Craig, 1999). A $\Delta sis1$ strain expressing *SIS1* from an *URA3*-based centromeric plasmid was transformed with a *sis1-121* mutant library in which the G/F sequences were randomly mutagenized by error prone PCR. We screened 5000 transformants, resulting in two independent mutants that produced normal levels of *Sis1-121* (our unpublished data), but did not support growth of $\Delta sis1$ cells on 5-FOA-containing media (Figure 4C). The mutations caused single amino acid alterations: Asn to Ile at position 108 (N108I) and Asp to Gly at position 110 (D110G), demonstrating that single amino acid substitutions in this stretch of amino acids unique to *Sis1* can disrupt its function. Thus, these amino acids found in the G/F region of *Sis1*, but not *Ydj1*, are not serving simply as a spacer between segments of the G/F, but are functionally important in their own right.

G/M Region of *Sis1* Is Functionally Redundant with the Unique Stretch of Amino Acids in G/F Region

The results discussed above demonstrate that the G/F region contains sequences that can be critically important for prion propagation. However, because *Sis1* contains a second glycine-rich region, the immediately adjacent G/M region, we wanted to test for any redundancy in function of these regions. First, we constructed a mutant containing a deletion of *Sis1*'s G/M region to test its ability to complement functions. Cells only containing *sis1* $\Delta g/m$ grew like wild-type cells. However, $\sim 5\%$ of *RNQ1* was consistently found in the

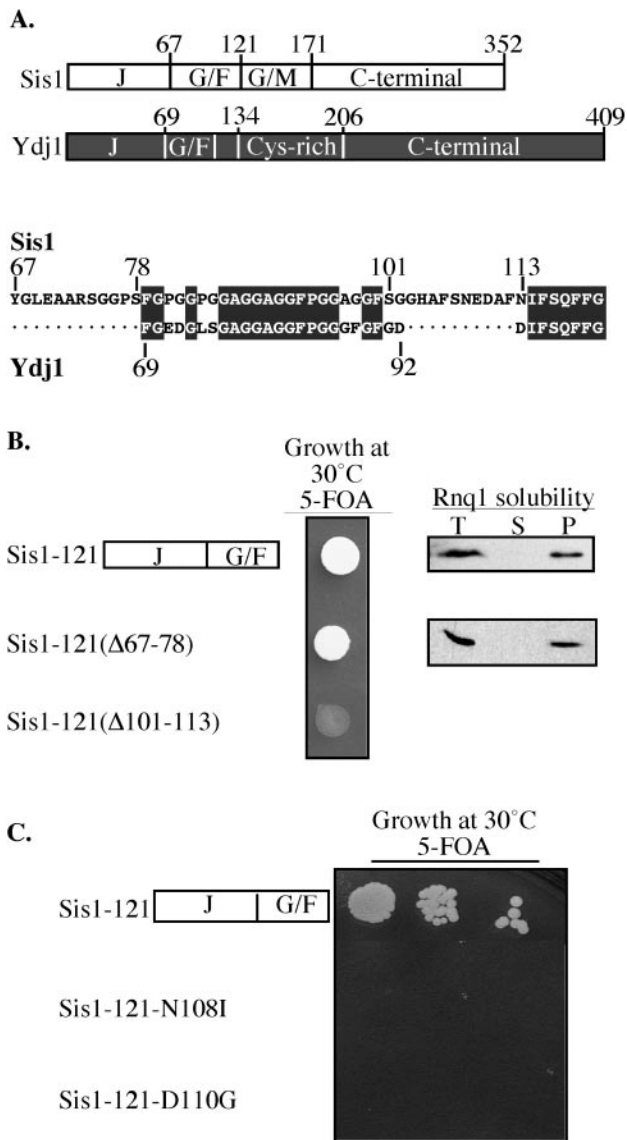


Figure 4. Unique residues within the G/F region of Sis1-121 are required for in vivo function. (A) Top, comparison of overall structure between Sis1 and Ydj1: J domain (J), glycine/phenylalanine region (G/F), glycine/methionine region (G/M)-rich region, and cysteine-rich region (Cys-rich). Bottom, sequence comparison of the G/F regions of Sis1 (top) and Ydj1(bottom) by using the Jotun Hein algorithm of the MegAlign software of the DNASTAR package (DNASTAR, Madison, WI). Identical residues are highlighted. Numbers refer to the position of the residues in the protein. (B) Left, cell suspensions of a $\Delta sis1$ strain expressing *SIS1* on a URA3-containing plasmid and a second plasmid expressing *sis1-121*, *sis1-121Δ67-78*, or *sis1-121Δ101-113* were spotted on medium containing 5-FOA and incubated for 2 d at 30°. Right, Rnq1 aggregation assays of lysates from $\Delta sis1$ cells expressing *sis1-121* or *sis1-121Δ67-78*. After centrifugation, equivalent aliquots of total lysate T, supernatant fraction S, and pellet fraction P were subjected to immunoblot analysis by using Rnq1-specific antibody. (C) Dilution series (1/10) of cell suspensions from a $\Delta sis1$ strain carrying *SIS1* on a URA3-based plasmid and a second plasmid expressing either Sis1-121 or Sis1-121 with two alterations (N108I or D110G) were spotted on media containing 5-FOA and incubated for 3 d at 30°C.

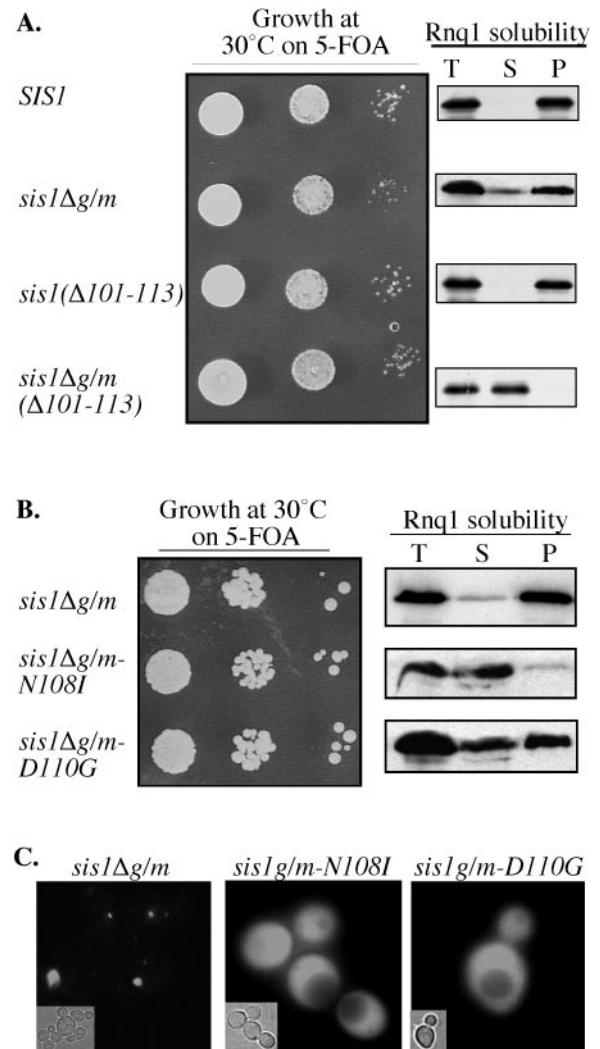


Figure 5. Unique residues within the G/F region of Sis1 are essential for the maintenance of $[RNQ^+]$ in the absence of the G/M region. (A and B) Left, 10-fold serial dilution series of cell suspensions from $\Delta sis1$ cells carrying *SIS1* on a URA3-based plasmid, as well as a second plasmid expressing wild-type or the indicated mutant were grown on medium containing 5-FOA for 3 d at 30°C. Right, Rnq1 aggregation assays were performed using lysates from $\Delta sis1$ cells expressing the specified *sis1* mutants. Equivalent amounts of total lysate (T), supernatant (S), and pellet (P) fractions from aggregation assays of lysates from the indicated strains were subjected to SDS-PAGE and analyzed for the presence of Rnq1 by immunoblot analysis. (C) Fluorescence microscopy of Rnq1-GFP in $\Delta sis1$ expressing *sis1Δg/m*, *sis1Δg/m-N108I*, or *sis1Δg/m-D110G* as described in Figure 2.

soluble fraction in aggregation assays (Figure 5A). No Rnq1 was found in the soluble fraction upon analysis of wild-type cells, suggesting that the G/M region may play a role in prion maintenance.

To further test this idea of functional redundancy, we combined alterations in the G/F region with deletion of the G/M region. First, a deletion of residues 101–113 in the context of otherwise full-length protein (Sis1 Δ 101-113) was

constructed. *Sis1* Δ 101-113 was able to maintain [RNQ⁺] in *Δsis1* cells (Figure 5A), consistent with the idea that both glycine-rich regions play a role in prion maintenance. The construct *sis1* Δ 101-113 Δ g/m sustained the growth of *Δsis1* cells similar to a wild-type strain, however, [RNQ⁺] was not maintained (Figure 5A). Moreover, single alterations of residues 108 or 110 in combination with a deletion of the G/M region resulted in either complete or significant loss of the prion, respectively, without affecting cell growth (Figure 5B). In aggregation assays using *Δsis1* lysates containing *Sis1* Δ G/M-N108I, Rnq1 was found only in supernatant fractions, whereas those containing *Sis1* Δ G/M-D110G resulted in 40% of the Rnq1 protein in supernatant fractions. These observations are consistent with analysis of fluorescence in cells expressing Rnq1-GFP. The fluorescence was dispersed in *Δsis1* cells expressing either *Sis1* Δ G/M-N108I or *Sis1* Δ G/M-D110G (Figure 5C). These results suggest an important role for unique residues of *Sis1* between positions 101 and 113 in prion maintenance. However, the G/M region complements the function of these residues.

Residues between Amino Acid 101 and 113 in *Sis1* Are Important for Propagation of Synthetic Prion [RPS⁺]

In the assays described above, no effect of the single amino acid alterations in the unique stretch between amino acids 101–113 on maintenance of [RNQ⁺] was found when in the context of the full-length protein, either in centrifugation assays (Figure 5A) or by observation of Rnq1-GFP fluorescence in vivo (our unpublished data). To test for more subtle effects on prion maintenance, we made use of an assay based on the ability of the prion conformation of Sup35, an essential yeast translation termination factor, to suppress the nonsense mutation *ade1-14* (Patino, 1996; Figure 6A). When Sup35 is in the prion form, [PSI⁺], nonsense suppression occurs due to a reduced efficiency of translation termination. We made use of the synthetic prion protein, Rmc, which is composed of the Rnq1 prion domain and the catalytic region of Sup35 (Middle and C-terminal region) and can substitute for Sup35 in vivo (Sondheimer and Lindquist, 2000). In the prion form, Rmc mediates the nonsense suppression of *ade1-14* cells, hence its designation as [RPS⁺], which stand for Rnq-PSI like-Suppression. This assay is very sensitive; lack of growth in the absence of adenine can result from minor changes in protein solubility that do not completely eliminate the prion conformation.

To validate the assay, 74-D694 *Δsis1* [RPS⁺] cells expressing *SIS1* or *sis1* Δ g/f were constructed and tested for the ability to grow in the absence of adenine. As expected 74-D694 *Δsis1* [RPS⁺] cells expressing *SIS1* from a plasmid were able to grow in the absence of adenine (Figure 6B) due to read-through of the nonsense codon. However, cells expressing *sis1* Δ g/f failed to grow in the absence of adenine, similar to [*rps*⁻] cells. Full-length *Sis1* having the alterations N108I and D110G were tested for their ability to maintain [RPS⁺] in 74-D694 cells. *sis1*N108I and *sis1*D110G did not support the growth of 74-D694 strain in the absence of adenine (Figure 6B), establishing the importance of these residues in a *Sis1* protein containing otherwise wild-type sequences for prion maintenance.

The essentiality of the unique G/F region sequences in [RPS⁺] maintenance allowed us to screen directly for alter-

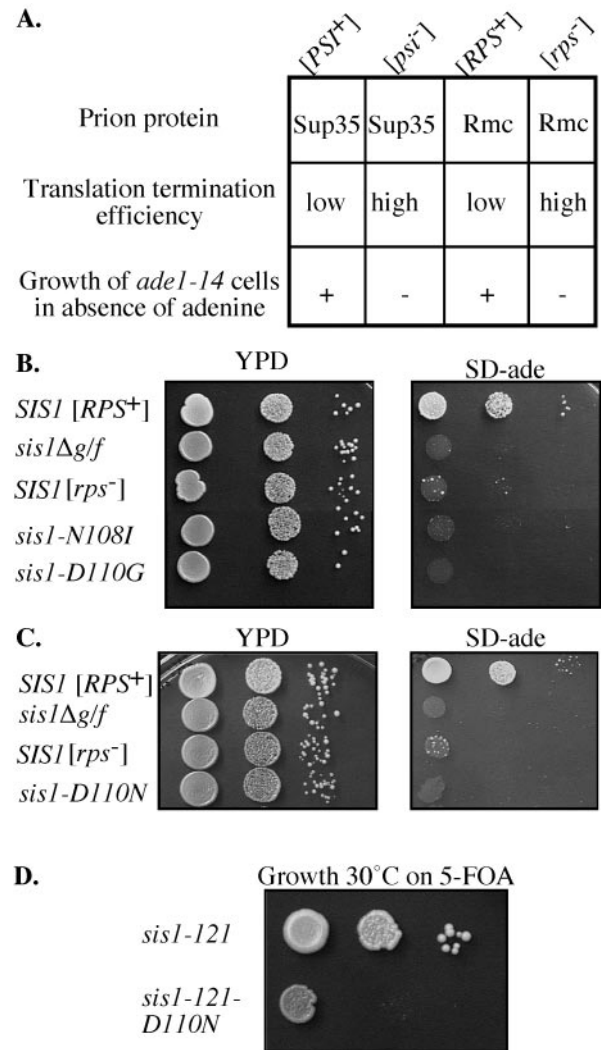


Figure 6. Unique residues in the G/F region of *Sis1* are important for maintenance of the prion [RPS⁺]. (A) Schematic representation of [RPS⁺]-based nonsense suppression. The translation terminator factor Sup35 is found in two protein states: prion state [PSI⁺] or the native state [psi⁻]. [PSI⁺] reduces the efficiency of translation termination resulting in nonsense suppression of the *ade1-14* (UAG) allele. Rmc is composed of the Rnq1 prion domain fused to the Middle and C-terminal catalytic domain of Sup35. The resulting synthetic prion, [RPS⁺], can suppress the *ade1-14* allele, hence its designation as Rnq1-PSI like-Suppression. (B and C) 74-D694 (*Δsis1* *Δsup35::RMC ade1-14* [RPS⁺]) cells expressing *SIS1* or various full-length *sis1* mutants. Serial dilutions (1/10) of cell suspensions of a *Δsis1* strain containing plasmids containing the designated genes. Growth was evaluated after 3 d on YPD or 5 d on adenine depleted medium at 30°C. (D) Growth of *Δsis1* W303 cells expressing either *sis1*-121 or the mutant *sis1*-121-D110N spotted onto YPD.

ations in the G/F region of the full-length gene that affected prion maintenance by screening for mutants that could not support growth on adenine. Efforts using this prion-based genetic screen identified a single mutation causing a substitution of Asn for Asp at position 110 (Figure 6C). Expression of *sis1*D110N allowed growth of 74-D694 cells in complete

medium but resulted in impaired growth in medium lacking adenine. Because this mutation was located in the same codon as another alteration (D110G) that affected the ability of Sis1-121 to support cell growth, we also tested whether the alteration D110N affected the capacity of *sis1-121* to support cell growth. A Δ *sis1* strain expressing *sis1-121D110N* grew poorly compared with cells expressing *sis1-121* (Figure 6D). Therefore, based on our genetic analysis, we conclude that the unique sequences of Sis1's G/F region are important both for supporting cell growth and prion maintenance.

DISCUSSION

The glycine-rich regions of Hsp40 J-type proteins, which frequently have been considered simply linker regions between the J domain and the unfolded protein-binding domain, are critical for the protein's function. Herein, we report that the sequences in this region are important for the specific function of the Sis1/Hdj1 Hsp40s (class II), and distinguish them from the Ydj1/Hdj2 Hsp40s (class I). Class I and II J-type proteins often function with Hsp70s as soluble proteins within the same cellular compartment. Under such circumstances a single Hsp70 may function with more than one Hsp40, thus being a part of a complex network of chaperones. An example of such cross-function is the interaction of both Sis1 and Ydj1 with the Ssa class of Hsp70s.

The results presented above demonstrate that residues in the G/F region of Sis1 play important roles in Sis1-specific functions required for cell viability, as well as maintenance of $[RNQ^+]$. Consistent with our hypothesis based on comparison of the G/F regions of Sis1 and Ydj1, a short sequence of amino acids found in Sis1, but not Ydj1, is required for both viability and prion maintenance. Therefore, this "insertion" in the G/F region of Sis1 is important for Sis1-specific function. However, this result does not mean that the conserved sequences in the G/F region do not play an important function in both Ydj1 and Sis1. The G/F region of Sis1 likely plays two roles, one generic and held in common with that of the G/F region of class I Hsp40s such as Ydj1, and the second specific for Sis1-type Hsp40s. This idea is consistent with the fact that overexpression of Sis1 can rescue growth defects of a *ydj1* strain (Caplan and Douglas, 1991), but overexpression of Ydj1 cannot compensate for the absence of Sis1 (Luke *et al.*, 1991).

Our observations demonstrate that Ydj1 does not complement Sis1's function in $[RNQ^+]$ maintenance. Nevertheless, it can interact with the prion form of Rnq1. Recently, it was reported that overexpression of Ydj1 can cure some variants of $[RNQ^+]$ (also known as $[PIN^+]$) (Bradley *et al.*, 2002), suggesting that Ydj1 antagonizes Sis1 in prion maintenance.

The sufficiency of the J domain and G/F region (Sis1-121) to support viability, as well as to maintain the prion form of Rnq1, facilitated dissection of the requirements for certain sequences in this truncated protein. The results of the analysis of full-length protein were more complex, revealing a redundancy in function within the glycine-rich region. The G/M portion of the glycine-rich region also plays a partially overlapping role with the G/F region. The single amino acid alterations in the G/F region that resulted in a nonfunctional Sis1-121 protein resulted in only mild defects when in the full-length protein. Similarly, deletion of only the G/M re-

gion had a modest effect on prion maintenance. However, in combination with the single amino alterations (N108I or D110G), the G/M deletion had dramatic effects on prion maintenance. Therefore, both sections of the glycine-rich region play a role in prion maintenance. Actually, the initial division of extended glycine-rich region into G/F and G/M segments was quite arbitrary. In fact, these two glycine-rich segments likely function together.

What does the glycine-rich region of Sis1 do? In vitro experiments indicate that deletion of the G/F region reduces the ability of an Hsp40 to enhance the interaction of an Hsp70 with a target protein (Wall *et al.*, 1995) (Lopez, Johnson, and Craig, unpublished data). Because Sis1, but not Ydj1, is required for maintenance of the Rnq1 prion, and the sequences responsible are within the glycine-rich region, we propose that Sis1 type molecular chaperones uniquely have the ability to facilitate the productive interaction of Hsp70 with particular substrates, including Rnq1. More generally, the glycine-rich regions of an Hsp40 may determine the specificity of interaction of an Hsp70 with substrate proteins. This idea is an expansion of the hypothesis of Rapoport and coworkers developed from the results of experiments in which the presence of only the J domain greatly enhanced the affinity of an Hsp70 for a variety of protein/peptide substrates (Misselwitz *et al.*, 1998). According to our expanded hypothesis, the J domain is required for this enhanced interaction with substrates, but the G/F region has evolved to determine the specificity of such interactions. In the case analyzed in our laboratory, the G/F region of the Sis1 family of Hsp40 can specifically enhance the productive interaction of Rnq1 with Hsp70.

However, it should be pointed out that glycine-rich regions are not required for function of all J-type proteins because members of class III, by definition, have no obvious region rich in glycine residues. Several members of that class have been found to function with Hsp70s at particular sites within organelles and some are important for targeting the Hsp70 to the site of function. Sec63 is localized to the ER membrane with a J domain located in the lumen targets Kar2/BiP to the translocation pore (Lyman and Schekman, 1995). Auxilin targets Hsp70 of mammalian cells to clathrin-coated vesicles where it functions in vesicle uncoating (Ungewickell *et al.*, 1995). Zuo1 functions with Ssb Hsp70 on yeast ribosomes as a chaperone for nascent chains (Yan *et al.*, 1998). G/F regions that may function to modulate the affinity of Hsp70 for particular substrates may not be as critical in cases where the Hsp70 is tethered to the location of the polypeptide substrate, thus dramatically increasing the local concentration. On the other hand, it is also possible that sequences, not recognizable to us because they are not rich in glycine residues, play a similar role.

An important goal of this study was to assess whether the role of Sis1 in prion maintenance was functionally different from its role in other cellular functions. This question was raised by the observation that Sis1 lacking the G/F region resulted in complete loss of the prion, but had little effect on cell viability. However, the analysis of mutants reported herein suggests that the sequence requirements are very similar, but that prion maintenance is particularly sensitive to slight alterations in Sis1 function. Therefore, we propose that biochemically Sis1 functions in prion maintenance as it

does in other cellular processes, although its exact role in prion maintenance remains to be elucidated.

The ability of the Sis1 homologs from both *Drosophila* and humans to not only carry out the essential function of Sis1 but also maintain the Rnq1 prion was surprising. Neither of these Hsp40s have any apparent sequence similarity with Sis1 in the glycine-rich regions, other than the general preponderance of glycine and phenylalanine residues that are also found in yeast Ydj1 and human Hdj2. Therefore, divergent sequences are able to perform the function carried out by amino acids 101–113 of Sis1. This divergence suggests that a certain structural conformation that can be manifested through a variety of interactions is required for class II Hsp40-specific function, rather than a requirement for specific amino acid contacts between the G/F region and other domains. Current research to determine whether the G/F sequences affect the structure of the adjacent J domain or directly interact with Hsp70 and alter its function is in progress.

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