An Essential Role for the Substrate-binding Region of Hsp40s in Saccharomyces cerevisiae

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Abstract. In addition to regulating the ATPase cycle of Hsp70, a second critical role of Hsp40s has been proposed based on in vitro studies: binding to denatured protein substrates, followed by their presentation to Hsp70 for folding. However, the biological importance of this model is challenged by the fact that deletion of the substrate-binding domain of either of the two major Hsp40s of the yeast cytosol, Ydj1 and Sis1, leads to no severe defects, as long as regions necessary for Hsp70 interaction are retained. As an in vivo test of this model, requirements for viability were examined in a strain having deletions of both Hsp40 genes. Despite limited sequence similarity, the substrate-binding domain of either Sis1 or Ydj1 allowed cell growth, indicating they share overlapping essential functions. Furthermore, the substrate-binding domain must function in cis with a functional Hsp70-interacting domain. We conclude that the ability of cytosolic Hsp40s to bind unfolded protein substrates is an essential function in vivo.

Key words: Ydj1 • Sis1 • molecular chaperone • heat shock protein 70 • yeast

Introduction
Hsp70s and Hsp40s cooperate in a wide range of cellular processes, including protein translation, translocation across membranes, and folding. Hsp70s bind unfolded or partially unfolded polypeptides in an ATP-regulated cycle (Hartl, 1996; Bukau and Horwich, 1998; Mayer et al., 2000). Hsp40s stimulate ATP hydrolysis, promoting the ADP-bound form of Hsp70, which has a higher affinity for unfolded protein substrates. In addition, many Hsp40s also bind unfolded polypeptide substrates and are able to prevent their aggregation independently of Hsp70 action. Based on the results of several in vitro studies, the current model of the cycle of Hsp70 and Hsp40 purports that Hsp40 first binds unfolded protein substrates and then transfers them to Hsp70 for folding (Langer et al., 1992; Liberek et al., 1995; Laufen et al., 1999).

Hsp40s are modular proteins. The signature J domain binds the ATPase domain of Hsp70 and is required for stimulating ATP hydrolysis. In class I and II Hsp40s, the J domain is followed by a region rich in glycine and phenylalanine residues (G/F) (Cheetham and Caplan, 1998).

Class I Hsp40s are distinguished by a cysteine-rich region that has been implicated in substrate binding (Banecki et al., 1996; Szabo et al., 1996). In addition, class I and II Hsp40s contain a poorly conserved COOH terminus that also has been implicated in binding unfolded polypeptides (Lu and Cyr, 1998a,b).

Multiple Hsp40s exist in both prokaryotic and eukaryotic cells. Here, we focus on two cytosolic Hsp40 chaperones of Saccharomyces cerevisiae, Ydj1 and Sis1 (see Fig. 1) (Caplan and Douglas, 1991; Luke et al., 1991). Lack of Ydj1, a class I Hsp40, results in a severe growth phenotype, especially at higher temperatures. Sis1 is an essential type II Hsp40. Some functional overlap exists between these two Hsp40s, as overexpression of SIS1 can partially rescue the growth defect of a ydj1 disruption strain. However, overexpression of YDJ1 does not restore viability to a sis1 disruption strain. Each of these Hsp40s is capable of stimulating the ATPase activity of the abundant essential cytosolic Hsp70, Ssa, and can cooperate with Ssa in refolding denatured luciferase (Lu and Cyr, 1998a,b).

Both Ydj1 and Sis1 can bind unfolded polypeptides. A proteolytic fragment of Ydj1 encompassing amino acids (a.a.) 179–384, which contains half of the cysteine-rich region and most of the COOH terminus, has been shown to bind denatured substrates in vitro (Lu and Cyr, 1998a). The region of Sis1 responsible for binding denatured substrates also maps to the COOH terminus (a.a. 171–352)
Materials and Methods

Yeast Strains

Strain JJ1146 (ydj1::HIS3) (Johnson and Craig, 2000) was crossed to the isogenic strain WY26 (sis1::LEU2) (Yan and Craig, 1999) to yield JJ1146 (MATa trp1-1 ura3-1 leu2-3,112 his3-11,15 ade2-1 can1-100 met2-1 lys2-Δ2 ydj1::HIS3 sis1::LEU2) (Yan and Craig, 1999). Strain JJ261 (MATa ade1-100 trpl1 ura3-52 leu2-3,112 ydj1::HIS3 ∆hem1) was constructed by crossing the nonisogenic strains MHI (MATa ura3-52 leu2-3,112 his4-519 (C-terminus), and dimerization domain (D). (B) Strain JJ1146 (sis1 ydj1/YCP50:SIS1) was transformed with indicated combinations of plasmids containing wild-type YDJ1 (or SIS1) and truncation mutants ydj1-134 (or sis1-121). After overnight incubation at 23°C, equal amounts of cells were plated onto selective media to maintain wild-type SIS1 (+SIS1) or media containing 5-FOA to counterselect for SIS1 (−SIS1). Plates were incubated at 23°C for 5 d.

Results and Discussion

The COOH Terminus of either Ydj1 or Sis1 Is Essential for Viability

The nonessentiality of the substrate-binding regions of Sis1 and Ydj1 in vivo contrasts with the proposed critical role of Hsp40s in binding denatured substrates before presentation of those substrates to Hsp70. Two possibilities come to mind. Either binding of unfolded protein substrates is not critical for Hsp40 function in the cytosol, or there is significant functional overlap between Sis1 and Ydj1, and either protein can carry out this function.

To test these ideas, we created a strain that contains chromosomal deletions of both YDJ1 and SIS1. Using combinations of plasmids expressing mutant and wild-type YDJ1 and SIS1 genes, we tested the importance of the COOH-terminal regions of these Hsp40s while maintaining the J plus G/F regions required for interaction with Hsp70. We constructed an sis1 ydj1 strain that carries a wild-type copy of SIS1 on a plasmid harboring URA3 to allow viability. To test for the ability of introduced genes to allow cell growth, transformants were spotted onto plates containing 5-fluoroorotic acid (5-FOA) to select for cells having lost the URA3-based plasmid containing SIS1. As expected from previous results (Yan and Craig, 1999), a strain carrying a combination of wild-type YDJ1 and sis1-121, encoding the J plus G/F regions, was viable (Fig. 1 B). Similarly, the combination of wild-type SIS1 plus ydj1-134, encoding the J
plus G/F plus spacer regions, was viable (Johnson and Craig, 2000). However, the combination of ydj1-134 did not support viability, even though the sequences necessary for Hsp70 interaction were retained. These results indicate that the COOH-terminal substrate-binding region of either Ydj1 or Sis1 is required in vivo, suggesting that Ydj1 and Sis1 may perform overlapping functions in the cell.

**Localization of the Essential Region in the COOH Terminus of Sis1**

Substrate binding is likely the essential function of the COOH terminus of Sis1, as a proteolytic fragment encompassing a.a. 171–352 is capable of binding denatured substrates in vitro (Lu and Cyr, 1998b). Recently, the crystal structure of this fragment revealed three domains (Fig. 2 C). Domains I and II have similar folds. However, domain I contains a prominent hydrophobic depression and thus was proposed to contain the substrate-binding site. In addition, the extreme COOH terminus (a.a. 338–352) is a dimerization domain (Sha et al., 2000).

To determine which sequences in the COOH-terminal fragment of Sis1 are essential in the absence of the COOH terminus of Ydj1, SIS1 truncation mutants were transformed into the sis1 ydj1 strain expressing ydj1-134. When transformants were spotted onto plates containing 5-FOA, colonies appeared expressing sis1-206, -253, -262, -297, and wild type (Fig. 2 A). However, when cells picked from these colonies were tested more carefully (Fig. 2 B, upper panel), it became apparent that cells expressing Sis1-206 grew slowly at 30°C, even though this truncation is expressed at wild-type levels (Fig. 2 B, lower panel). Cells expressing Sis1-253, -262, or -297 exhibited wild-type growth rates.

These results clearly indicate that domain II (a.a. 260–336) and the dimerization region (a.a. 338–352) of Sis1 are not required for normal growth at 30°C even in the absence of the COOH terminus of Ydj1. However, domain I (a.a. 180–255) is required, as sis1-206 cells grow very slowly, and sis1-171 cells are inviable in this assay. Sha et al. (2000) proposed that, within domain I, residues V184 and L186 from strand 1, F201, and I203 from strand 2, and F251 from strand 5 interact with bound substrate (Fig. 2 C), however, the role of these residues in substrate interaction has not yet been established. Sis1-262 and Sis1-253 contain these residues, whereas Sis1-206 truncates after strand 2 and thus lacks F251 and β sheets that are probably important in stabilizing the domain. Our results suggest that Sis1-262 contains a functional substrate-binding domain. However, at this time, the only available in vitro assay of Sis1 chaperone function measures the ability to bind and refold denatured luciferase in cooperation with Hsp70 (Sha et al., 2000). Since activity in these in vitro assays is dependent on the presence of the dimerization domain of Sis1, we are unable to monitor the in vitro substrate-binding activity of Sis1-262 in the absence of domain II and the dimerization domain. However, our genetic results demonstrate a critical role of domain I in the absence of the COOH terminus of Ydj1 and are consistent with that role being substrate binding.

**Localization of the Essential Regions in the COOH Terminus of Ydj1**

Having found the region of Sis1 that is critical in the absence of the COOH terminus of Ydj1, we asked the same question of Ydj1: what portion of Ydj1 is essential in the absence of the COOH terminus of Sis1? The substrate-binding region of Ydj1 has been localized between a.a. 179 and 384 of Ydj1 (Lu and Cyr, 1998a), a fragment that contains part of the cysteine-rich region and most of the COOH terminus (Fig. 1 A). The extreme COOH terminus...
formed with wild-type or media containing 5-FOA to counterselect for After overnight incubation at 23°.

Plates were grown at 23°C. (A) Colonies that appeared on selective media after 2 d at 30°C (left). Growth of ydj1 mutants on selective media after 2 d at 30°C in a ydj1 disruption strain (WT SIS1) (right). WT, wild-type.

of Ydj1 contains the CAAX box, the site of a farnesylation modification thought to be involved in localization of Ydj1 to membranes (Caplan et al., 1992b). Truncation and point mutants of YDJ1, picked because they disrupt function of these regions, were transformed into the sis1 ydj1 strain that carried wild-type SIS1 on a URA3-based plasmid and also expressed sis1-121. The shortest truncation that allowed growth in the absence of full-length Sis1 was ydj1-274, which contains not only the J plus G/F region, but also the cysteine-rich region and 68 a.a. of the COOH terminus (Fig 3 A). When tested for growth at 30°C, it was clear that ydj1-274 allowed cells to grow nearly as well as wild-type YDJ1.

These results are consistent with substrate binding being a critical role of the region distal to the J plus G/F. Mutant DnaJ proteins having alterations in the cysteine-rich region have shown defects in suppression of aggregation of rhodanase and binding to some, but not all, protein substrates (Banecki et al., 1996; Szabo et al., 1996). Therefore, the cysteine-rich region is implicated in binding of at least some protein substrates. However, a fragment containing only the J plus G/F plus cysteine-rich region of Ydj1 ( ydj1-206) did not support viability when sis1-121 was the only copy of SIS1 present. This result suggests that, in addition to the cysteine-rich region, the first 68 a.a. of the COOH terminus are required for the ability to bind substrates in vivo. Thus, our results are in agreement with studies in which a fragment of Ydj1 encompassing a.a. 179–384 bound denatured substrate (Lu and Cyr, 1998a).

Other mutants that could be recovered on 5-FOA did not grow as well as ydj1-274 (Fig 3 B). Although sequences required for substrate binding or other sequences important in allowing proper transient interactions with substrates have not been clearly established, the amino acid substitution G315D led to in vivo defects in binding of glucocorticoid receptor (Kimura et al., 1995). In vitro, G315D was found to be defective both in suppressing rhodanase aggregation and refolding denatured luciferase (Lu and Cyr, 1998a). Consistent with a role in substrate interaction, an amino acid substitution at the same residue, ydj1-G315E, exhibited severe growth defects when sis1-121 was the only SIS1 gene present (Fig 3 B). Although the most straightforward interpretation of these results is that G315 and surrounding residues are important for substrate binding, it is also possible that, in light of the robust growth of ydj1-274, COOH-terminal sequences can act negatively on more proximal residues, inhibiting their ability to function. Consistent with this idea, the truncation mutant ydj1-363, which results in removal of fewer residues from the COOH terminus than ydj1-274, and ydj1-C406S, which alters the farnesylation signal, grow poorly in the presence of sis1-121 compared with the truncation ydj1-274. The C406S mutation has been shown to result in a decrease in the membrane association of Ydj1 (Caplan et al., 1992b). The associated temperature-sensitive growth defect was attributed to this alteration in cellular localization. However, in other proteins, the farnesylation modification has been shown to not only affect cellular localization but also protein–protein interactions (Sinensky, 2000); so it is possible that farnesylation has additional effects on Ydj1 function. Further studies will be required to determine how the farnesylation modification of Ydj1 affects in vivo chaperone activity.

Figure 3. Essential region of the COOH terminus of Ydj1. Strain J1146 ( ydj1 sis1/YCp50SIS1) expressing sis1-121 was transformed with wild-type YDJ1 or the indicated ydj1 mutants. (A) After overnight incubation at 23°C, equal amounts of cells were plated onto selective media to maintain wild-type SIS1 (+ SIS1) or media containing 5-FOA to counterselect for SIS1 ( SIS1). Plates were grown at 23°C for 5 d. (B) Colonies that appeared on 5-FOA plate (sis1-121) were grown overnight and serial dilutions were grown at 30°C for 2 d on selective media (left). Growth of ydj1 mutants on selective media after 2 d at 30°C in a ydj1 disruption strain (WT SIS1) (right). WT, wild-type.

The COOH Terminus of Sis1 Can Substitute for the COOH Terminus of Ydj1 In Vivo, but Only in cis

The results presented above indicate that substrate binding is critical for Hsp40 function. In the current model of interaction between substrates, Hsp40 and Hsp70, substrate first bind the COOH terminus of Hsp40 and then are targeted to Hsp70 via interaction of the J domain with the ATPase domain of Hsp70 (Langer et al., 1992; Liberek et al., 1995; Laufen et al., 1999). The J domain of both Ydj1 and Sis1 have been shown to be important in vivo, as a single amino acid change in either J domain has severe effects on function. In a ydj1 disruption strain, an F47L alteration of Ydj1 causes both severe growth defects and defects in the maturation of Hsp90 substrates (Johnson and Craig, 2000). The H34Q mutation of Sis1 disrupts the HPD motif in the J domain and causes a null phenotype in an sis1 disruption strain (Yan and Craig, 1999).

We conducted in vivo tests to determine whether a functional J domain and COOH-terminal substrate-binding domain must be part of the same protein (Fig. 4). Growth of an sis1 ydj1 strain expressing sis1-121 and ydj1-134 could not be rescued by either H34Q Sis1 or F47L Ydj1. Nor did the COOH-terminal fragment of Sis1 (a.a. 170–352) expressed in trans with J plus G/F allow viability. These results indicate that the COOH terminus of Sis1
must be present in the context of a wild-type J domain to function, indicating that close cooperation between the two domains is essential for function in vivo. However, in support of the idea that the COOH-terminal regions can substitute for one another, a fusion protein (YYS-262) that joins the G/M region and domain I of Sis1 to the J plus G/F of Ydj1 (Yan and Craig, 1999) allows viability (Fig. 4).

**YYS Fusion Proteins Partially Rescue Hap1 Defect In Vivo**

Sis1 and Ydj1 have important functions in the cell, but few of their specific in vivo roles are known. Little is known about Sis1 function, although it has been reported to be required for the initiation of translation (Zhong and Arndt, 1993). Ydj1 cooperates with the Hsp70 Ssa in the translocation of preproteins into the ER and mitochondria (Caplan et al., 1992a; Becker et al., 1996). In addition, Ydj1 has been shown to be required for the in vivo maturation of the heterologous proteins v-src and steroid receptors via the Hsp90 pathway (Kimura et al., 1995; Fliss et al., 1999; Johnson and Craig, 2000), but native substrates have been elusive. However, recent evidence suggests that the transcription factor Hap1, which regulates the expression of *CYC1* and other genes encoding respiratory functions in a heme-dependent manner (Zitomer and Lowry, 1992), is such a substrate. When heme levels are low, Ydj1 and the yeast Hsp90, Hsp82, bind Hap1, and Hap1 activity is low. When heme levels increase, Ydj1 and Hsp82 dissociate from Hap1, and its transcriptional activity increases. Hsp82 is believed to play a role in the activation of Hap1, as decreased levels of Hsp82 led to a large decrease in the overall activity of a Hap1 reporter gene construct without affecting Hap1 levels (Zhang et al., 1998).

To determine whether Ydj1 also plays a role in the activation of Hap1, we examined Hap1 activity in *ydj1* mutant strains containing a *hem*1 mutation, which allowed manipulation of heme levels. Varying amounts of the heme precursor, ALA, were added to the media to maintain high or low levels of heme. Hap1 activity was monitored by measuring transcription from the UAS1 of the *CYC1* promoter linked to the *lacZ* gene (Guarente et al., 1984). In low levels of ALA (4 μg/ml), strains expressing mutant or wild-type *YDJ1* had similarly low levels of β-galactosidase activity, 1–4 U (data not shown). In the presence of high concentrations of ALA (250 μg/ml), cells expressing wild-type *YDJ1* had dramatically higher activity, 343 U. In contrast, cells expressing the J plus G/F region of Ydj1 (*ydj1*-104) had only 30 U of activity (Fig. 5 A), indicating that...
deletion of the substrate-binding region of Ydj1 causes an activation defect of Hap1, resulting in >10% of the activity observed in wild-type cells.

Since the substrate-binding region of Ydj1 is required for activity of Hap1, we were able to directly test whether the substrate-binding region of Sis1 is able to replace Ydj1 in this function. For this, we used fusion proteins that join the J plus G/F of Ydj1 to the G/M region and domain I of Sis1 (Fig. 5 A). In the absence of ydj1, expression of SIS1 from a high copy plasmid restored wild-type levels of Hap1 activity. In addition, expression of YYS-262 partially restored Hap1 activity (180% β-galactosidase U), whereas cells expressing YYS-206 exhibited the same level of Hap1 activity as ydj1-I-104. Thus, YYS-262 is able to substantially rescue a specific defect, that is loss of Hap1 activity that arises in the absence of the substrate-binding region of Ydj1.

To determine if YYS-262 could more generally carry out Ydj1 functions, we tested its effect on growth of a ydj1 strain at 30°C and 34°C. As previously reported, the ydj1-I-104 construct partially rescues growth at 30°C but exhibits a severe temperature-sensitive phenotype (Johnson and Craig, 2000). YYS-262 allowed near wild-type levels of growth at 30°C and 34°C (Fig. 5 A), but not 37°C (data not shown). YYS-206 could not support growth at 34°C, supporting evidence from Fig. 2 that this truncation of domain I is not fully functional. Thus, in addition to rescuing the Hap1 defect, domain I of the Sis1 COOH terminus can rescue the growth defect that arises in the absence of the substrate-binding region of Ydj1.

Summary

The COOH termini of Sis1 and Ydj1 share overlapping essential roles. In addition, the J domain of Ydj1 may substitute for the J domain of Sis1 (Yan and Craig, 1999). In contrast, the essential function of the G/F region of Sis1 cannot be replaced by the G/F region of Ydj1 (Yan and Craig, 1999), indicating a unique role of the Sis1 G/F region. Although the substrate binding domains of Sis1 and Ydj1 can substitute for one another under some conditions, they share only 29% sequence identity, compared with 66% sequence identity between the J domains. This difference likely results in some differences in substrate specificity, which may be responsible for the temperature-sensitive phenotype observed in the absence of the substrate-binding region of either Ydj1 or Sis1. Further studies will be needed to understand the specificity of Hsp40 interactions with Hsp70.

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