A Conserved HPD Sequence of the J-domain Is Necessary for YDJ1 Stimulation of Hsp70 ATPase Activity at a Site Distinct from Substrate Binding

Joyce Tsai‡ and Michael G. Douglas§

From the Department of Biochemistry and Biophysics, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

The 46-kDa protein YDJ1 is one of several known yeast homologues of the Escherichia coli DnaJ protein. Like all J homologues, it shares homology with the highly conserved NH₂-terminal “J-domain” of DnaJ. A component of the DnaK (Hsp70) chaperone machinery that mediates protein folding, DnaJ is necessary for survival at elevated temperatures. It stimulates ATP hydrolysis by DnaK and effects the release of DnaK-bound polypeptides. Previous genetic and biochemical studies indicate that the J-domain is necessary for these functions. Using peptides corresponding to J-domain sequences, we show that a peptide containing the highly conserved His-Pro-Asp sequence at positions 34–36 in the J-domain competes off YDJ1 stimulation of Hsp70 ATPase activity. Inhibitory concentrations of peptide do not prevent binding of folding substrates, therefore YDJ1 must interact with Hsp70 at a site distinct from that for substrate binding. This interaction is critical for Hsp70 activity, since a mutant YDJ1 protein harboring a H34Q change (ydj1Q34) stimulates neither Hsp70 ATPase nor substrate release. The importance of the proper function of this region of the protein is supported by the poor growth and temperature-sensitive phenotype of yeast expressing ydj1Q34.

The heat shock family of proteins (Hsps)¹ include members now known to function as molecular chaperones. These Hsps bind nascent polypeptides as they emerge from cytosolic ribosomes (Beckmann et al., 1990; Nelson et al., 1992), escort fully translated proteins to their destinations in cellular organelles, maintain them in a transport-competent partially unfolded state (Deshaies et al., 1990), and are necessary on both sides of the membrane for the efficient transport of proteins into mitochondria (Vogel et al., 1990; Scherer et al., 1990; Nguyen et al., 1991). The roles of chaperones in protein folding have been extensively studied (for recent review, see Hartl et al. (1992)). There are two major chaperone families identified as mediators in protein folding. First, the Hsp70s bind short (7–9-amino acid) sequences of extended polypeptides (Flynn et al., 1991). Second, the Hsp60s bind to emergent secondary structures on folding proteins (Landry and Gierasch, 1991). Neither the Hsp70s nor the Hsp60s act alone; they require co-chaperones, which modulate their activities (Georgopoulos et al., 1990).

YDJ1 is a co-chaperone protein in Saccharomyces cerevisiae which modulates the activity of Hsp70 (Cyr et al., 1992). It shares extensive homology to the Escherichia coli protein DnaJ and is one of several homologues of DnaJ identified in yeast. Each of the homologues has evolved to occupy a specific niche in the eukaryotic cell. Whereas YDJ1 is largely cytosolic and endoplasmic reticulum membrane-associated (Caplan and Douglas, 1991), SIS1 is predominantly nuclear and cytosolic ribosome-associated (Luke et al., 1991), and CAJ1 is membrane-associated and appears to bind calmodulin (Mukai et al., 1994). Another J homologue, XDJ1, is either a silent gene or transcribed under unknown conditions (Schwarz et al., 1994). The common feature of all of these proteins which define them as J homologues is homology to the NH₂-terminal 80 amino acids of DnaJ protein. This sequence is defined as the J-domain. Whereas SEC63, 20tatin, and CAJ1 share only this J-domain, the other yeast homologues, including YDJ1, share homology and structural features with DnaJ elsewhere in the protein as well (Caplan et al., 1993). Presumably, each eukaryotic J homologue is specialized to perform, within its cellular environment, one or a few of the many known functions of DnaJ. Of the yeast homologues, YDJ1 and SCJ1 are most closely related to DnaJ (Caplan et al., 1993). However, YDJ1 is distinguished among the homologues by being farnesylated (Caplan et al., 1992b).

The prototype J homologue, DnaJ, was first identified as a gene product necessary for λ-phage replication in E. coli and has been cloned and sequenced (Bardwell et al., 1986; Ohki et al., 1986). Since its initial characterization, DnaJ has been shown to act in conjunction with the E. coli Hsp70 prototype, Dnak, and a third protein, GrpE. Together, this trio participates in such diverse functions as λ-phage replication (Ospiuk et al., 1993; Hoffmann et al., 1992), plasmid P1 replication (Sonhammer and Chatteraj, 1993; Wickner et al., 1992), chromosomal DNA replication (Hupp and Kaguni, 1993), folding of nascent polypeptides (Hendrick et al., 1993), export of fully translated polypeptides from the bacterium (Wild et al., 1992), the repair of heat-induced protein damage (Schroder et

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‡ Present address: Neurobiology Dept., Swiss Federal Institute of Technology, CH8093 Zürich, Switzerland.

§ To whom correspondence should be addressed: Sigma Diagnostics, 545 S. Ewing, St. Louis, MO 63103. Tel.: 800-521-8956; Fax: 314-531-2858.

¹The abbreviations used are: Hsp(s), heat shock protein(s); Fmoc, N-(9-fluorenyl)methoxycarbonyl; HPLC, high performance liquid chromatography; DTT, dithiothreitol; MOPS, 3-(N-morpholino)propanesulfonic acid; PCR, polymerase chain reaction; CMLA, carboxymethylalactalbumin.
al., 1993; Ziemienowicz et al., 1993), and the assembly of macromolecular complexes in flagellum synthesis (Shi et al., 1992). In the folding of nascent polypeptides and denatured proteins, DnaK binds and releases extended hydrophobic regions, preventing protein misfolding (Langer et al., 1992). Each cycle of binding and release is dependent upon ATP hydrolysis, at which DnaK is slow. DnaJ stimulates the ATP hydrolytic activity of DnaK, allowing a completed cycle of peptide binding and release, whereas GrpE acts as a nucleotide exchanger, preventing protein misfolding (Langer et al., 1992). Recent genetic and biochemical evidence supports the long standing idea that the conserved J-domain of DnaJ and its homologues mediates interaction with DnaK and cognate Hsp70s. Mutations in this region prevent function of SEC63 in conjunction with the endoplasmic reticulum Hsp70, Kar2 (Scidmore et al., 1993). In E. coli, characterization of the dnaJ259 mutant that cannot support -phage replication (Sell et al., 1990) revealed a single amino acid change in a highly conserved region within the J-domain. The NH2-terminal 108 amino acids of DnaJ alone, containing the full J-domain, are sufficient to support -phage replication and to stimulate DnaK ATP hydrolysis (Wall et al., 1994).

Previous work from this laboratory demonstrated that YDJ1 stimulates the ATPase activity of and polypeptide substrate release from its most likely cytosolic cognate Hsp70, SSA1 (Cyr et al., 1992). In the present study, we have used synthetic peptides corresponding to J-domain sequence to compete with purified YDJ1 protein to ask which regions specifically interact with Hsp70 to stimulate these activities. One such region was identified, mutagenized, and tested in for in vitro effects and in vitro activity.

MATERIALS AND METHODS

Peptides—Peptides were synthesized in the University of North Carolina/Program in Molecular Biology and Biotechnology microprotein chemistry facility located in the Department of Microbiology, University of North Carolina School of Medicine, using Fmoc chemistry in a Rainin (Woburn, MA) Symphony multiple peptides synthesizer. Polyethylene glycol-poly styrene resins and appropriately protected Fmoc amino acids were purchased from Millipore (Milford, MA) on a 10-mm hydroxyapatite as described above. Purified YDJ1 or ydj1Q34 was then dialyzed against buffer, and bound proteins were eluted using a 5–400 mM sodium phosphate gradient. Peak fractions were pooled, concentrated, and dialyzed against 50 mM HEPES, pH 7.4, 10 mM DTT, 10% glycerol (buffer A), aliquoted, and snap frozen in liquid N2 before storage at −80 °C.

YDJ1 and ydj1Q34 were purified as described previously (Cyr et al., 1992) from BL21(DE3). E. coli (Novagen, Madison, WI) containing pET9dYDJ1 (Caplan and et al., 1992b) and pET9dYQPD, respectively. Cells were grown in LB + kanamycin at 37 °C until A600 = 1.0. Isopropyl-1-thio-β-D-galactopyranoside was added to 0.5 mM, and induction proceeded for 2 h, after which cells were harvested and resuspended in ice-cold 20 mM MOPS, pH 7.5, 10 mM DTT, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10.0 μM leupeptin, and 10.0 μM pepstatin. After cell disruption by sonication, the lysate was cleared by centrifugation at 100,000 × g and loaded onto DE52 equilibrated with lysis buffer. The column was washed with 3 column volumes of buffer, and elution was performed using a 0–500 mM NaCl gradient over 20 volumes. Purified protein was dialyzed against buffer A and separated using hydroxypatite as described above. Purified YDJ1 or ydj1Q34 was then dialyzed against buffer B, aliquoted, and snap frozen in liquid N2 before storage at −80 °C.

YDJ1 in BL21(DE3) was described previously (Cyr et al., 1992b). General yeast methods followed those in Guthrie and Fink (1991). For overexpression in and purification from E. coli, a 1.3-kilobase PCR product comprising the open reading frame of ydj1Q34 was cloned in-frame behind the T7 promoter, into the Neo+ and BamHI sites of the pBluescript SK II vector (Stratagene). The product was transformed into BL21(DE3), which contains the T7 polymerase gene under the control of the lacUV5 promoter (Studier et al., 1990). The resulting strain overexpressed ydj1Q34 upon induction with isopropyl-1-thio-β-D-galactopyranoside, as roughly 30% of total protein. Overexpression of wild type YDJ1 in BL21(DE3) was described previously (Caplan et al., 1992b). General molecular biology methods followed those in Sambrook et al. (1989).

ATP Hydrolysis and Gel Shift Assays—To assay the ATPase activity of Hsp70 in vitro, 50-μl reactions contained 10 mM HEPES, pH 7.4,
peptides, only p21–40 significantly reduced YDJ1-stimulated Hsp70 ATPase activity (Fig. 2 and Table I). 0.5 μM SSA1 or 0.5 μM SSA1 + 1.0 μM YDJ1 were incubated with increasing amounts of either p1–20 or p21–40. At a 1:1 ratio of p21–40 to YDJ1, ATPase activity was reduced by roughly 50%. At a 50:1 ratio, only 21% of the initial YDJ1-stimulated increase in ATPase activity remained. By contrast, neither p1–20 (Fig. 2) nor the other two peptides reduced SSA1 ATPase activity (Table I). Therefore p21–40 includes a J-domain sequence necessary for interaction with Hsp70ssa1, which alone is capable of competing with full-length YDJ1 for that interaction. None of the peptides alone could stimulate Hsp70 ATPase activity (Fig. 2 and data not shown).

To identify candidate sequences within p21–40 which might mediate interaction with Hsp70, we considered both sequence conservation and the context within the predicted protein structure. Although the entire J-domain is quite highly conserved, the amino acids at positions 34–36 (HPD) are absolutely conserved between DnaJ and all of the known yeast homologues. Moreover, this sequence is situated in a loop or hinge region between the two α-helices that were initially predicted after sequence analysis of DnaJ (Bardwell et al., 1986). These helices might serve to present a conserved loop sequence for interaction with other proteins. To test the possibility that this conserved sequence could interact with Hsp70, three synthetic peptides from this region were used to compete with YDJ1p. A 4-mer with the sequence YHPD could not compete off YDJ1-stimulated ATPase activity. We examined the possibility that sequences within amino acids 21–32 were responsible for the reduction in ATPase stimulation by using the 20-mer pYHPDX_{16} in competition experiments. This peptide, corresponding to amino acids 33–52, exhibited a weak ability to compete compared with p21–40. This weak competition could not be attributed to amino acids 41–52, since p41–60 did not compete. Because amino acids 21–32 were not present on this peptide and because residues 37–41 are not well conserved, it seemed plausible that the HPD sequence was responsible for this competition. It was reasoned that the position of the conserved sequence within a competing peptide might be important. The 10-mer X_{8}YHPDX_{5} containing the candidate sequence nestled in the center was slightly better in competition experiments.

**RESULTS**

**The Hinge Region between Helices 2 and 3 of the J-domain Mediates Interaction with Hsp70.**—We initially used synthetic peptides representing different regions of the J-domain of YDJ1 to compete with YDJ1p for interaction with Hsp70ssa1. These experiments would identify regions within the J-domain which are necessary for stimulation of Hsp70. Four peptides that were 20 amino acids long were synthesized to span the first 80 amino acids of YDJ1: p1–20, p21–40, p41–60, and p61–80 (Fig. 1). Each of these peptides was incubated with purified Hsp70ssa1 in the presence and absence of YDJ1 under assay conditions to ascertain which peptides, if any, might compete off YDJ1-stimulated Hsp70 ATPase activity and/or stimulate ATPase activity in the absence of YDJ1. Of the four
YDJ1 J-Domain Interactions with Hsp70

FIG. 2. Ability of p21–40 to reduce YDJ1-stimulated Hsp70 ATP hydrolysis. 0.5 μM purified SSA1 or 0.5 μM SSA1 and 1.0 μM YDJ1 was incubated with 0.0, 1.0, 10.0, 20.0, and 50.0 μM p1–20 or p21–40 in the presence of Mg \(^2+\) and [α-\(^32\)P]ATP. ADP formation was determined after a 15-min incubation at 30 °C by spotting a 2-μl aliquot onto polyethyleneimine-cellulose, chromatographic separation, and quantitation of radioactivity in ADP- and ATP-containing spots. Results were normalized with respect to total counts in each sample and adjusted for spontaneous ATP hydrolysis. Neither p1–20 nor p21–40 stimulated SSA1 ATPase activity. However, p21–40 reduced ATP hydrolysis in the samples containing YDJ1, indicating that p21–40 might be competing with YDJ1 for interaction with SSA1 and that a sequence within amino acids 21–40 of the J-domain mediates this interaction. By contrast, no competition was observed for p1–20.

Table I

Effect of peptides on YDJ1-stimulated SSA1 activity

Peptides representing wild type and mutant sequences within the YDJ1 J-domain and COOH terminus were tested for their abilities to compete off YDJ1-stimulated Hsp70 SSA1 ATPase activity. In paired experiments, 0.5 μM SSA1 was incubated with 1.0 μM YDJ1 in the absence (SSA1 + YDJ1) and presence (+Peptide) of up to 50 or 100 μM competing peptide using assay conditions described under “Materials and Methods.” Normalized average cpm of ADP released for three separate experiments sampled in duplicate are shown. At the maximum peptide concentration for each set of experiments the percent activity remaining was calculated using the YDJ1-stimulated increase over basal SSA1 ATPase activity in the absence of peptide as 100%.

| Competitor | SSA1 + YDJ1 | +Peptide | Concentration | Activity |
|------------|-------------|----------|---------------|----------|
| p1–20      | 2,995       | 3,180    | 50            | 100      |
| p21–40     | 2,990       | 626      | 50            | 21       |
| p41–60     | 2,945       | 3,430    | 50            | 100      |
| p61–80     | 2,945       | 3,043    | 50            | 100      |
| YHPD       | 15,492      | 15,726   | 100           | 100      |
| X_YHPDx₁₅  | 15,492      | 10,875   | 100           | 70       |
| X_YHPDx₃   | 15,492      | 13,606   | 100           | 88       |
| p21–40H3A4Q| 4,519       | 2,957    | 50            | 65       |
| pSASQ      | 5,940       | 6,237    | 100           | 100      |
| pC-farnesy| 5,993       | 6,597    | 100           | 100      |

Table II

Effect of peptides on YDJ1-stimulated Hsp70 SSA1 ATPase activity

Increasing amounts of peptides YHPD, YHPDx₁₅, and X_YHPDx₃ were added to samples containing 0.5 μM SSA1 and 1.0 μM YDJ1. Samples were incubated for 15 min at 30 °C, after which [α-\(^32\)P]ADP formation was assessed. An aliquot from each sample was removed for separation by thin layer chromatography and quantitation. Results were normalized for total counts in each sample, and the nonstimulated SSA1 activity was subtracted. The 4-mer was unable to compete with YDJ1 for interaction with SSA1. Somewhat longer HPD-containing peptides, however, could interact. Inclusion of a 100 molar excess of YHPDx₃ reduced the YDJ1-stimulated SSA1 ATPase activity by 12%. Likewise, the 10-mer X_YHPDx₃ reduced the rate of ADP hydrolysis by 30%.

C of the CaaX box) is changed to serine. This substitution results in a TS phenotype (Caplan et al., 1992b) and defective transport of polypeptide precursors into organelles in vivo (Caplan et al., 1992a). pC-farnesy|corresponds to the farnesylated and proteolytically processed COOH terminus of YDJ1. Neither peptide showed any detectable competition of YDJ1-stimulated Hsp70 ATPase activity, even at peptide:YDJ1 molar ratios of 100:1.

J-domain and Hsp70 Interact at a Site Distinct from That for Polypeptide Substrate Binding—Although p21–40 was observed to reduce the YDJ1-stimulated ATPase activity of Hsp70, this could result from the peptide being bound as substrate and not by competing off YDJ1 stimulation. Different polypeptide substrates of Hsp70 have been observed to reduce ATPase activity, presumably by forming a stable complex, or to increase ATPase activity.³ We addressed whether p21–40 could compete with CMLA, a known synthetic substrate of Hsp70, for binding and also whether p21–40 could compete off the YDJ1-stimulated release of bound CMLA (Fig. 4). 0.0, 10.0, and 100.0 molar ratios of p1–20 and p21–40 were added to reactions containing 1.0 μM \(^{125}\)I-CMLA and 0.5 μM SSA1 with or without 1.0 μM YDJ1. After incubation, samples were separated by nondenaturing electrophoresis, and SSA1-bound CMLA was visualized by autoradiography and quantitated by densitometry. In these studies, p21–40 at molar ratios well above those that inhibit YDJ1-stimulated ATPase activity did not compete with \(^{125}\)I-CMLA for binding to the Hsp70 substrate site. Although 10 μM p1–20, 100 μM p1–20, and 10 μM p21–40 seemed to reduce Hsp70-bound CMLA (lanes 3, 5, and 7), this result was small and not always reproducible. Moreover, there was no further decrease of CMLA-SSA1 complex formation as the p21–40 concentration was increased to 100 μM (lane 9). This consistency in complex formation is not mirrored by constancy of CMLA release. Ten μM p1–20 had essentially no effect upon release (lane 4), although increasing the concentration of peptide to 100 μM did stabilize the CMLA-SSA1 complex (lane 6). Significantly, as little as 10.0 μM competing peptide p21–40 effectively blocked release of bound substrate

³ J. Tsai, L. Estey, and M. G. Douglas, submitted for publication.
Characterization of YDJ1 J-Domain Interactions with Hsp70

To compare the ability of wild type YDJ1 and the mutant protein ydj1Q34 to stimulate SSA1, 0.5 μM purified SSA1 was incubated under assay conditions with either 0.5, 1.0, 2.0, or 5.0 μM ydj1Q34 or the same concentrations of ydj1Q34 and YDJ1. Samples were incubated for 15 min at 30 °C, after which a 2-μl aliquot was removed. [α-32P]ADP and [α-32P]ATP were separated by thin layer chromatography and quantitated. Values were normalized with respect to total counts in each sample. Because the ydj1Q34 preparation contained a small amount of contaminating ATPase, the values for ADP formation in control samples containing only the indicated concentrations of ydj1Q34 in reaction buffer were subtracted from each set of experimental samples. The mutated protein ydj1Q34 lacked the ability to stimulate Hsp70 ATPase even when presented in 10-fold molar excess.

Characterization of ydj1Q34—To address directly the contribution of a single amino acid in the conserved HPD sequence to J-domain interaction with Hsp70, the ydj1Q34 allele was subcloned into an E. coli inducible expression vector and purified. Upon addition of up to 5.0 μM ydj1Q34 to 0.5 μM SSA1, there was essentially no increase in ATPase activity (Fig. 5). This is in contrast to the activity seen upon the addition of wild type YDJ1 protein, with which SSA1 ATPase activity achieved maximal stimulation by 1.0 μM.

The ability of ydj1Q34 to stimulate substrate release and to compete with YDJ1 for substrate release was also examined. Ability to compete without ability to stimulate would indicate that binding of YDJ1 alone is not sufficient for the conformational change in SSA1 which results in peptide substrate release. Fig. 6 shows that although a 2:1 molar ratio of YDJ1:SSA1 effects release of bound 125I-CMLA (lane 2), the same ratio of ydj1Q34 could not stimulate any release (lane 3). However, this inability does not stem from a total inability to bind to SSA1p. We observed that a 10-fold excess of ydj1Q34 could prevent YDJ1 stimulation of CMLA release from Hsp70hap1 (lane 4).

These results indicate two possibilities: either that ydj1Q34 possesses a weaker affinity for Hsp70 hap1 or that ydj1Q34 possesses wild type affinity but that upon binding the mutant ydj1Q34 protein does not stimulate ATPase activity and substrate release. To distinguish between these two possibilities, we used peptide p21–40H34Q in competition experiments (Fig. 7). p21–40H34Q mimics the amino acid change in ydj1Q34 and is otherwise identical to p21–40. If ydj1Q34 affinity for SSA1 remains the same as that of the wild type YDJ1, then p21–40H34Q and p21–40 (wild type) should compete off the YDJ1-stimulated ATPase activity of SSA1 equally well. The addition of 50 μM p21–40 to a reaction mixture containing 0.5 μM SSA1

**Fig. 5. Comparison of YDJ1 and ydj1Q34 stimulation of Hsp70 ATPase.** To compare the ability of wild type YDJ1 and the mutant protein ydj1Q34 to stimulate SSA1, 0.5 μM purified SSA1 was incubated under assay conditions with either 0.5, 1.0, 2.0, or 5.0 μM ydj1Q34 or the same concentrations of ydj1Q34 and YDJ1. Samples were incubated for 15 min at 30 °C, after which a 2-μl aliquot was removed. [α-32P]ADP and [α-32P]ATP were separated by thin layer chromatography and quantitated. Values were normalized with respect to total counts in each sample. Because the ydj1Q34 preparation contained a small amount of contaminating ATPase, the values for ADP formation in control samples containing only the indicated concentrations of ydj1Q34 in reaction buffer were subtracted from each set of experimental samples. The mutated protein ydj1Q34 lacked the ability to stimulate Hsp70 ATPase even when presented in 10-fold molar excess.

**Fig. 4. Peptide competition gel shift experiments.** Nondenaturing gel electrophoresis and autoradiography were used to evaluate 125I-CMLA-SSA1 complexes in the presence of J-domain peptides (top panel). The star (*) marks the position of the CMLA-SSA1 complex. 1.0 μM radioiodinated CMLA was incubated with 0.5 μM purified SSA1 in the presence of Mg-ATP, forming a 125I-CMLA-SSA1 complex (lane 1). Inclusion of 1.0 μM YDJ1 stimulates release of CMLA from SSA1 (lane 2). J-domain peptides do not prevent formation of CMLA-SSA1 complex, as there is no significant decrease of complex with increasing peptide addition (lanes 3 and 5, 7, and 9). When added to samples containing YDJ1 as well, 10 μM p1–20 could not compete off the YDJ1-stimulated CMLA (lane 4), although 100 μM p1–20 could do so to some extent (lane 6). By contrast p21–40 appears to have a reproducible stabilizing effect on the complex at both 10 μM and 100 μM concentrations (lanes 8 and 10). These results were quantitated and confirmed by densitometry (bottom panel). Numbered lanes correspond to those lanes scanned on the autoradiogram above. The amount of 125I-CMLA complex present in the absence of either peptide or YDJ1 (lane 1) was taken as 100% bound.

(lane 8), and a large majority of CMLA remained bound to SSA when p21–40 was increased to 100.0 μM (lane 10). These results clearly indicate that p21–40 interacts specifically with SSA1 to compete off substrate release but does not affect substrate binding. They also demonstrate that YDJ1 must interact with SSA1 at a site distinct from that of polypeptide substrate binding. YDJ1 stimulates substrate release by a mechanism that does not include displacement of polypeptide substrate from Hsp70.

**Characterization of JOY4**—During the course of these experiments, Wall et al. (1994) reported that the NH2-terminal 108 residues of DnaJ containing the entire J-domain are both necessary and sufficient for interaction with DnaK. They also characterized the dnaJ259 mutant, which was deficient in both stimulation of DnaK activity and also in support of λ-phage replication. This mutant contained a histidine to glutamine change at residue 33, within the highly conserved HPD sequence. To examine the significance of this conserved region in the eukaryote, we created the analogous mutant in S. cerevisiae. Amino acid 34 was changed from histidine to glutamine with a base substitution using PCR-based site-directed mutagenesis, creating the allele ydj1Q34 (see “Materials and Methods”). This allele was introduced into a ydj1 null background, resulting in JOY4. This strain was temperature-sensitive, exhibiting slow growth at 30 °C and no growth at 37 °C. The JOY4 doubling time in rich liquid media at 30°C was 6 h, and there was no detectable growth in minimal liquid media (data not shown).

**Characterization of ydj1Q34**—To address directly the contribution of a single amino acid in the conserved HPD sequence to J-domain interaction with Hsp70, the ydj1Q34 allele was subcloned into an E. coli inducible expression vector and purified. Upon addition of up to 5.0 μM ydj1Q34 to 0.5 μM SSA1, there was essentially no increase in ATPase activity (Fig. 5). This is in contrast to the activity seen upon the addition of wild type YDJ1 protein, with which SSA1 ATPase activity achieved maximal stimulation by 1.0 μM.
and 1.0 μM YDJ1 decreased maximal ATP hydrolysis by 72%; however, the addition of 50 μM p21-40H34Q achieved only a 35% reduction. These and the data in Fig. 6 suggest that ydj1Q34 binds to SSA1 with less affinity than wild type YDJ1 and that the ydj1Q34-SSA1 interaction is not transduced into an effect on DnaK conformation relative to wild type DnaJ. The analogous to dnaJ259, which was shown to have reduced influence on DnaK conformation relative to wild type DnaJ. The conformational change in Hsp70 protein upon interaction with wild type J-proteins would result in two different populations of Hsp70 molecules, reflecting those that had interacted with J-proteins and those that had not. This may explain why we never observed complete reduction of SSA1 ATP hydrolysis. Although p21-40H34Q reduced YDJ1-stimulated ATPase activity to 65% of that in the absence of peptide, it could not compete off YDJ1 as effectively as the wild type sequence.

In this study, the peptide p21-40 competes with YDJ1 for stimulation of SSA1 ATPase activity and CMLA release but does not prevent formation of the CMLA-SSA1 complex. This argues against any model that proposes that substrate release from Hsp70s results from direct displacement by J-proteins. It is possible, however, that J-protein interaction and peptide substrate binding may still be mutually exclusive because of differing conformations of Hsp70 for binding of either. Although p21-40 could not prevent binding of CMLA to SSA1 at concentrations that prevented release of bound CMLA, the 20-mer is unlikely to have had any effect on SSA1 conformation, since it could not alone stimulate SSA1 ATPase activity. Ydj1Q34 was able to interact with SSA1 to prevent YDJ1-stimulated release of CMLA in gel shift experiments; however, it too is unlikely to affect SSA1 conformation since it could not alone stimulate ATPase activity or peptide release. This is analogous to dnaJ259, which was shown to have reduced influence on DnaK conformation relative to wild type DnaJ. The conformational change in Hsp70 protein upon interaction with wild type J-proteins would result in two different populations of Hsp70 molecules, reflecting those that had interacted with J-proteins and those that had not. This may explain why we never observed complete reduction of SSA1 ATPase activity to basal levels in the presence of YDJ1 and a large excess of the competing peptide p21-40.

It is likely that other regions of the J-domain are also sites of interaction with Hsp70s. In our study, shorter HPD-containing peptides could not compete off YDJ1-stimulated Hsp70 ATPase activity, indicating that other residues must be necessary for YDJ1-Hsp70 binding. It was surprising that none of the other
three J-domain peptides, p1–20, p41–60, and p61–80, exhibited any measurable effect on the YDJ1-stimulated increase in SSA1 ATPase activity, despite the presence of very highly conserved sequences throughout the J-domain spanned by these peptides. Recent publication of the the NMR structure for amino acids 2–108 of DnaJ suggests a model that accounts for the importance of both the HPD sequence in the interhelical hinge region and the selective pressure for sequence conservation along the rest of the J-domain. Two groups (Szyperski et al., 1994; Hill et al., 1995) found independently that in contrast to the predicted structure of two α-helices, the nonmonomeric J-domain possesses four helices that interact strongly with one another to form a hydrophobic core. The two longer helices 2 and 3 associate with one another to present the conserved HPD in the interhelical loop. In this model, the sequence conservation in the helical regions of the J-domain preserves the precise interactions that determine the tertiary structure of the domain, which is necessary for presentation of the HPD sequence. While this manuscript was in review, several nonconserved positions within the J-domain were also identified as necessary for binding to Hsp70. These nonconserved residues serve to determine the specificity of J homologues for different Hsp70 molecules (Schlenstedt et al., 1995). Taken together, these data on the roles of conserved and nonconserved residues within the J-domain suggest that tertiary conformations are important for the presentation of specific residues necessary for recognition, binding, and stimulation. The 20-mer peptides p1–20, 41–60, and 61–80 are too short to attain these conformations. By contrast, p21–40 largely corresponds to a loop region. Because the J-domain is monomeric in solution, p21–40 cannot prevent YDJ1 stimulation of Hsp70 by disruption of the YDJ1 dimer.

That several nonconserved residues have been identified as necessary for J homologue binding to specific cognate Hsp70s suggests that the absolutely conserved HPD sequence is specifically required for stimulation of ATPase activity. This conclusion is supported by our demonstration that the ydj1Q4 mutant protein can prevent YDJ1-stimulated release of bound CMLA by Hsp70 \(^{4}\), despite its total lack of ability to stimulate either ATPase activity or substrate release on its own. The mutant protein was able to prevent CMLA release at lower ratios relative to YDJ1 than those required for competition by p21–40 peptide (Figs. 4 and 6 and data not shown), thus suggesting that the full-length protein is capable of binding but not stimulating Hsp70. This ability of ydj1Q4 to bind Hsp70 nonproductively, as well as its solubility and purification characteristics, which are identical to those of the wild type YDJ1 protein, confirms that the mutant is not dysfunctional because of any misfolded structure.

It is noteworthy that amino acids 22–29 in the DnaJ from Clostridium acetobutylicum, KKAFLKLA (Behrens et al., 1993), are similar to a sequence of Raf kinase, RKTFLKLA, which may interact with γ-phosphate residues of ATP.\(^4\) Amino acids 23–30 of YDJ1, KKYAEKCA (Caplan and Douglas, 1991) and 22–29 of E. coli DnaJ, KKYKRLA (Bardwell et al., 1986; Ohki et al. 1986) are less similar; however, it is tantalizing to speculate that this sequence present in helix 2 of the J-domain and represented in the competing peptide p21–40 may also have a role in regulating Hsp70 activity. If this is the case, then J-proteins would interact with Hsp70s at or near their catalytic ATPase domain.

It is now confirmed that the J-domain of DnaJ and a eukaryotic homologue, YDJ1, are necessary to effect a conformational change in Hsp70 leading to increased ATPase activity and peptide substrate release. What then, is the function of the remainder of the YDJ1 molecule, which shares other conserved domains with DnaJ? Szyperski et al. (1994) have shown that the glycine/phenylalanine-rich stretch immediately COOH-terminal to the J-domain of DnaJ is flexible in solution, possibly to allow proper orientation of the J-domain for interaction with DnaK. The zinc finger domain, containing four repeats of the motif CXXCXXGXXC, has an unknown function. Although DnaJ does function in DNA replication, there has been no demonstration of DNA binding capabilities. Moreover, this motif is conserved in the cytosolic protein YDJ1. Caplan et al. (1992a) have shown genetically that YDJ1 functions as a dimer; possibly this domain serves as a dimerization site. YDJ1 associates with membranes upon heat shock in a farnesylation-dependent manner (Caplan et al., 1992b); however, this farnesylation appears to be a signal for translocation upon heat shock and may not confer intrinsic affinity for membranes.

The zinc fingers might be analogous to the “zinc butterflies” described for protein kinase C and Raf kinase, which bind phospholipids and phospholipids. This region has been shown to be farnesylated and, like YDJ1, is necessary for translocation to membranes and survival at elevated temperatures. It is likely that the COOH-terminal region of YDJ1 is specialized for YDJ1’s role as a cytoplasmic chaperone that also associates with organelar membranes to deliver protein substrates.

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