Regulation of Hsp70 Function by a Eukaryotic DnaJ Homolog*

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We report that a purified cytoplasmic Hsp70 homolog from Saccharomyces cerevisiae, Hsp70SSA1, exhibits a weak ATPase activity, which is stimulated by a purified eukaryotic dnaJp homolog (YDJlp). Stable complex formation between Hsp70SSA1 and the constantly unfolded protein carboxymethylated α-lactalbumin (CMLA) was assayed by native gel electrophoresis. The affinity of Hsp70SSA1 for CMLA appeared to be regulated by YDJlp. Significant reduction in both CMLA-Hsp70SSA1 complex formation and the release of CMLA pre-bound to Hsp70SSA1 was observed only in the presence of both YDJlp and ATP. Thus, Hsp70SSA1 and YDJlp interact functionally in the execution of Hsp70SSA1 chaperone activities in the eukaryotic cell.

Molecular chaperones of the Hsp70 family bind polypeptide substrates to stabilize or alter their conformation and hydrolyze ATP to facilitate polypeptide release (1). Intracellular processes in which Hsp70 family members participate include protection of cells from thermal stress, protein folding and assembly, disassembly of protein complexes, protein degradation, protein trafficking, and the initiation of bacteriophage DNA replication in Escherichia coli (for reviews see Refs. 1–3).

Several reports indicate that the activity of the Hsp70 family members is regulated. In E. coli, Hsp70 (dnaKp) functionally interacts with two other heat shock proteins, dnaJp and grpEp, in bacteriophage λ and P1 DNA replication (3). Purified dnaJp and grpEp synergistically stimulate the ATPase activity of dnaKp; dnaJp stimulates ATP hydrolysis by dnaKp, whereas grpEp stimulates adenine nucleotide exchange (4). dnaKp, dnaJp, and grpEp can also act sequentially to enhance in vitro protein folding catalyzed by the chaperoningroEL (Hsp60; Ref. 5). In eukaryotes, unidentified N-ethylmaleimide-sensitive factor(s) act with cytosolic Hsp70 molecules to maximally stimulate in vitro protein transport into mitochondria (6), the endoplasmic reticulum (7), and the cell nucleus (8).

The recent identification of dnaJ homologs in eukaryotes suggests they participate in at least some of the reactions catalyzed by eukaryotic Hsp70 family members (9–15). In Saccharomyces cerevisiae different dnaJ homologs are localized to the same subcellular compartments as the different Hsp70 members; YDJ1 is cytosolic (9, 10), SIS1 partitions between the cytosol and cell nucleus (11), Sec63 is found in membranes of the endoplasmic reticulum (12, 13), and SCJ1 is found in mitochondria (14). The YDJ1 gene encodes the more abundant of the two cytosolic dnaJp homologs and is required for normal cell growth (9, 10). YDJlp is farnesylated at a C-terminal CaaX box (where a is an aliphatic amino acid and X is any residue), and this modification appears to mediate partitioning of YDJlp between the cytosol and different intracellular membranes (9, 16). To test for interactions between the abundant eukaryotic Hsp70 family members and dnaJ homologs, YDJlp and a cytosolic Hsp70 homolog of S. cerevisiae, Hsp70SSA1, were purified. The influence of YDJlp on Hsp70 ATPase activity and polypeptide substrate binding and release were then examined. The results of such experiments are reported below.

EXPERIMENTAL PROCEDURES

Purification of YDJlp—YDJlp was overexpressed in E. coli strain BL21 (DE3) as described previously (16). Cells from a 200-ml culture were resuspended in 10 volumes of ice cold buffer A (20 mM MOPS, pH 7.5, 0.5 mM EDTA, 10 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride) and then disrupted by sonication. The lysate was cleared by centrifugation at 100,000 × g for 30 min. The S100 was loaded directly onto a DE52 column (1.0 × 5.0 cm, Whatman) equilibrated with buffer A at 4 °C. The column was washed with 10 volumes of buffer A and bound YDJlp was eluted with a 0–300 mM NaCl gradient. Peak fractions containing YDJlp were pooled and then dialyzed against buffer B (5 mM potassium phosphate, pH 7.0, 10 mM DTT). YDJlp was next loaded onto a hydroxyapatite column (1 × 5 cm, Bio-Rad) equilibrated with buffer B at 4 °C. The column was washed with 10 volumes of buffer B, and bound YDJlp was eluted with a 5–400 mM potassium phosphate gradient. Peak fractions were pooled and then dialyzed against buffer C (10 mM Hepes, 50 mM NaCl, 10 mM DTT, and 10% glycerol), concentrated, snap-frozen in liquid nitrogen, and stored at −70 °C. Protein concentrations were determined using the Bio-Rad Bradford assay kit with bovine serum albumin as the standard.

Purification of an Hsp70 Fraction Enriched in SSA1p—S. cerevisiae strain MW141 (Mata, ura3-52, leu2-3, 112, his3-11, 15, ssa1::HIS3, ssa2::LEU2, ssa4::URA3, pGAL1-SSA1) was grown at 30 °C in 6 liters of YP media supplemented with 2% galactose to induce high level expression of Hsp70SSA1. Cells were harvested at OD600 = 10. The cell pellet (50 g, wet weight) was resuspended in 50 ml of 500 mM NaCl, 50 mM Hepes (pH 7.4), 10 mM DTT, 2 mM MgCl2, 0.5 mM EDTA, 19 μM leupeptin, 10 μM pepstatin, and 1 mM phenylmethylsulfonyl fluoride. Cells were disrupted by agitation with glass beads (6 pulses of 1-min duration, which were followed by a 10-min cooling period) using a Beadbeater (Biospec). Hsp70 molecules present in the S100 of the lysate were purified by chromatography on ATP-agarose (C-8 linked, Sigma) and DE52 (Whatman) columns as described previously (17, 18). As a final purification step Hsp70 was loaded onto a hydroxyapatite column (1.5 × 10 cm, Bio-Rad) and eluted with a 5–400 mM potassium phosphate gradient. Peak fractions were pooled and then dialyzed against buffer C, concentrated, snap-frozen with liquid nitrogen, and stored at −70 °C.

Assay of Hsp70SSA1 ATPase Activity—Purified Hsp70SSA1 was in-
cubated in reaction mixtures containing 50 mM Heps, pH 7.4, 50 mM NaCl, 10 mM DTT, 2 mM MgCl₂, and ATP (as indicated, [α-²³P] ATP, 7.0 × 10⁷ to 1.0 × 10⁹ cpm/pm). Reaction mixtures were set up on ice and shifted to 30 °C for the specified time. Reactions were then placed on ice, and duplicate 2-µl aliquots were assayed for ADP formation by thin layer chromatography on polyethyleneimine-cellulose plates (19). Spontaneous ADP formation was also assayed and subtracted prior to calculations for rates of ATP hydrolysis. The pH and salt conditions employed were optimized for maximal stimulation of Hsp70SSA ATPase activity by YDJ1p.

**Gel Shift Assay for ¹²⁵I-CMLA Binding to Hsp70SSA**—Binding reactions were carried out at 30 °C for 20 min in 20-µl reaction mixtures composed of the following: Hsp70SSA (2.5-3.0 µM), 50 mM Heps, pH 7.0, 50 mM NaCl, 10 mM DTT, 0.1 mM EDTA, 0.4% bovine serum albumin, and ¹²⁵I-CMLA (0.7 µM, 4.5 × 10⁸ cpm/pm). The concentrations of indicated reagents were: YDJ1p (0.3-4 µM), 2 mM MgCl₂, 1 mM ATP, 1 mM AMP-PNP. After incubation, reaction mixtures were diluted 2-fold with ice-cold 2 × reaction buffer made 20% (v/v) in glycerol and 0.01% in bromophenol blue. Diluted samples were loaded directly onto a 10-15% linear gradient native gel and run on ice at 10-20 mA. After electrophoresis, gels were immediately fixed, stained with Coomassie Brilliant Blue R-250, dried, and then used to expose x-ray film. The gel mobility shift of ¹²⁵I-CMLA migration was specific for Hsp70SSA as bovine serum albumin, YDJ1p, and enolase, respectively, at 5 µg/reaction mixture had no effect on CMLA migration. The Hsp70SSA and ¹²⁵I-CMLA concentrations used are in the linear range for complex formation. To calculate binding in percent of control bands on the gel corresponding to ¹²⁵I-CMLA and ¹²⁵I-CMLA-Hsp70SSA complex formation was observed, 10-30% of ¹²⁵I-CMLA added to reaction mixtures formed a complex with Hsp70SSA. However, no variation in the level of complex formation was observed in assays of duplicate reaction mixtures on the same gel. The level of complex formation observed has been previously documented in gel filtration assays, which monitor complex formation between other Hsp70 homologs and CMLA (5, 20).

**CMLA Isolation**—¹²⁵I-CMLA was made by labeling 100 µg of carboxymethylated lactalbumin (CMLA, Sigma) with 0.5 mCi of carrier-free NaI (ICN) using IODO-GEN (Pierce Chemical Co.) as described by the manufacturer.

**RESULTS**

Purified Hsp70 and YDJ1p used in this study were obtained from two different sources. An Hsp70 fraction highly enriched in SSA1p (Hsp70SSA) was purified from S. cerevisiae strain MW141, which was genetically engineered to constitutively express only SSA1, and not the other three SSA genes, which encode cytosolic Hsp70 homologs (6). YDJ1p was overexpressed and purified from E. coli. YDJ1p is farnesylated in S. cerevisiae (16), but since E. coli lacks protein isoprenyl transferases, purified YDJ1p used in this study was not farnesylated. Both protein preparations were greater than 98% pure (Fig. 1).

To test for interactions between Hsp70SSA and YDJ1p, the influence of YDJ1p on Hsp70SSA ATPase activity was determined. Hsp70SSA hydrolyzed ATP at a rate of 2-5 nmol/mg/min depending on the protein preparation. These rates are typical of other Hsp70 homologs (4, 18, 21). YDJ1p exhibited no detectable ATPase activity (not shown). However, addition of YDJ1p to reaction mixtures stimulated Hsp70SSA ATPase activity approximately 10-fold at all time points tested (Fig. 2A). The fold stimulation of Hsp70SSA ATPase activity by YDJ1p was constant over a range of ATP concentrations which were above and below the Kₐ of Hsp70SSA for ATP (approximately 2.5 µM). Thus, YDJ1p influences the maximal velocity of the ATPase reaction and not the affinity of Hsp70SSA for ATP (Fig. 2B). Maximal stimulation of Hsp70SSA ATPase activity was observed at a YDJ1p:Hsp70SSA molar ratio near 1.0 (Fig. 2C). Preincubation of YDJ1p for 10 min at 70 °C prior to assay reduced stimulation of Hsp70SSA ATPase activity by YDJ1p 90% (not shown), indicating that the native conformation of YDJ1p must be recognized in order for the two proteins to interact productively.

Conformational changes in Hsp70 due to ATP hydrolysis have been correlated with release of bound polypeptides from Hsp70 homologs (1, 18, 21, 22). To test the influence of the ATPase stimulatory molecule, YDJ1p, on polypeptide binding to Hsp70SSA, a gel shift assay was developed to monitor stable interactions between Hsp70SSA and polypeptide substrates. Since the affinity of Hsp70 for native proteins is low (20, 22), reduced and carboxymethylated α-lactalbumin (CMLA), which is permanently unfolded, was labeled with ¹²⁵I and utilized as a substrate (5, 20). ¹²⁵I-CMLA migrated with high mobility on a native polyacrylamide gel (Fig. 3A, lane 1). Incubation with YDJ1p had no influence on ¹²⁵I-CMLA migration (Fig. 3A, lane 1 versus 2). However, incubation with Hsp70SSA shifted migration of ¹²⁵I-CMLA to a position coincident to that of Hsp70SSA, indicating the proteins form a stable complex (Fig. 3A, lane 1 versus 3). ¹²⁵I-CMLA-Hsp70 complex formation was essentially unchanged in the presence of ATP or YDJ1p alone (Fig. 3A, lane 3 versus 4 and 5). However, the combination ATP and YDJ1p reduced Hsp70SSA-CMLA complex formation by over 70%. When ATP was replaced by AMP-PNP, a non-hydrolyzable ATP analog, YDJ1p had no significant influence on ¹²⁵I-CMLA-Hsp70 complex formation (Fig. 3, lane 3 versus 8), indicating that YDJ1p-dependent reductions in complex formation were coupled to ATP hydrolysis.

To determine if YDJ1p stimulates release of pre-bound ¹²⁵I-CMLA from Hsp70SSA1, binding assays were carried out in two steps. In the first step Hsp70SSA and ¹²⁵I-CMLA were incubated to allow complex formation. In the second step, the reaction mixture containing the ¹²⁵I-CMLA-Hsp70SSA1 complex was split and incubated further. Addition of ATP to the second reaction resulted in release of 20% of the CMLA bound to Hsp70SSA1 in the first reaction (Fig. 3B, lane 1 versus 2), whereas addition of YDJ1p had no effect. Inclusion of both YDJ1p and ATP in the second incubation resulted in dissociation of 60% of the complex (Fig. 3B, lane 1 versus 4). Thus, the combination of YDJ1p and ATP not only prevents substrate binding to Hsp70SSA1 (Fig. 3A) but can also stimulate substrate release from the molecule.

CMLA is an artificial substrate of Hsp70 and not capable of folding after release from the chaperone. This prompted us to test an alternative substrate for binding to Hsp70SSA. When a peptide that is specifically recognized by the mitochondrial import apparatus (27), Flb1-51, was employed as a substrate, stable complex formation with Hsp70SSA was
were incubated for 20 min at 30 °C. For details pertaining to the composition of reaction mixtures and assay of ADP formation see "Experimental Procedures." Hsp70SSA1 ATPase activity in a dose-dependent manner; Hsp70SSA1 and at least two different protein substrates. 8, stimulation of Hsp70SSA1 ATPase activity by YDJlp is independent of ATP concentration; Hsp70SSA1 (0.30 μM), YDJlp (0.30 μM), and ATP (as indicated, 1.4 × 10^5 cpm/pm) were incubated in 20-μl reaction mixtures at 30 °C for 5 min. C, YDJlp stimulates Hsp70SSA1 ATPase activity in a dose-dependent manner; Hsp70SSA1 (0.25 μM), ATP (50 μM, 7.0 × 10^5 cpm/pm), and YDJlp (as indicated) were incubated for 20 min at 30 °C. For details pertaining to the composition of reaction mixtures and assay of ADP formation see "Experimental Procedures."

observed.2 As with CMLA (Fig. 3A) the combination of ATP and YDJlp was required to effect significant substrate release,2 indicating that YDJlp regulates the interactions between Hsp70SSA1 and at least two different protein substrates. ATP-dependent reduction of 125I-CMLA-Hsp70 complex formation by YDJlp was, however, never complete. The small amount of 125I-CMLA that remained bound might be due to either the formation of a nonspecific complex or due to a specific association in which the Hsp70SSA1 molecule could either not respond to YDJlp or which may require an additional component for complete release. To establish the specificity of 125I-CMLA-Hsp70SSA1 complex formation, unlabeled competitor CMLA was included in reactions to determine if the level of CMLA bound to Hsp70SSA1 could be reduced below that measured in the presence of YDJlp and ATP (Fig. 4A, lane 2). At a 100-fold molar excess of unlabeled CMLA, the Hsp70SSA1-CMLA complex was reduced to only 22% of control valves. This was the same level of residual binding observed

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2. D. M. Cyr and M. G. Douglas, unpublished observation.

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**Fig. 2. YDJlp stimulates Hsp70SSA1 ATPase activity.** A, Kinetics of Hsp70SSA1 ATPase activity. Hsp70SSA1 (0.52 μM) and YDJlp (0.67 μM), as indicated, were incubated with ATP (100 μM; 1.8 × 10^5 cpm/pm) in 60-μl reaction mixtures at 30 °C. At the specified time, duplicate 2-μl aliquots were removed and assayed for ADP formation. B, stimulation of Hsp70SSA1 ATPase activity by YDJlp is independent of ATP concentration; Hsp70SSA1 (0.30 μM), YDJlp (0.30 μM), and ATP (as indicated, 1.4 × 10^5 cpm/pm) were incubated in 20-μl reaction mixtures at 30 °C for 5 min. C, YDJlp stimulates Hsp70SSA1 ATPase activity in a dose-dependent manner; Hsp70SSA1 (0.25 μM), ATP (50 μM, 7.0 × 10^5 cpm/pm), and YDJlp (as indicated) were incubated for 20 min at 30 °C. For details pertaining to the composition of reaction mixtures and assay of ADP formation see "Experimental Procedures."
Hsp70SSA1 but as a regulator. 1) Stoichiometry data (Figs. 1C and 4B) indicate that equimolar YDJ1p concentrations are required to stimulate ATP hydrolysis maximally and also to reduce polypeptide–Hsp70SSA1 complex formation. 2) YDJ1p-dependent reduction of Hsp70-CMLA complex formation requires ATP hydrolysis (Fig. 3A). 3) YDJ1p stimulates ATP-dependent release of substrates pre-bound to Hsp70SSA1. In other independent studies, we observed that YDJ1p does not appear to be a general substrate for Hsp70 homologs since it does not stimulate the ATPase activity of purified dnaKp or BIP at concentrations which stimulate Hsp70SSA1 ATPase activity maximally. Furthermore, interactions between YDJ1p and Hsp70SSA1 do not appear as stable as those between polypeptide substrates and Hsp70SSA1 since no alteration in the mobility of 125I-CMLA–Hsp70SSA1 complex is observed on native gels upon addition of YDJ1p to reaction mixtures (Fig. 3A, lane 3 versus 6). The lack of stable YDJ1p binding to Hsp70SSA1 raises questions about the nature of Hsp70 and dnaJ homolog interactions in vivo. Do dnaJ homologs form stable complexes with Hsp70 family members to permanently stimulate ATPase activity and cycling on and off polypeptide substrates? Alternatively, do dnaJ homologs interact transiently with Hsp70 family members to stimulate ATP hydrolysis and polypeptide release at specific subcellular locations where discharge of bound polypeptide substrates is required for entry into protein folding or protein translocation pathways? The present data support the alternative. YDJ1p stimulates ATP-dependent release of substrates pre-bound to Hsp70SSA1 (Fig. 3B) and is preferentially localized to membranes (9, 16) where Hsp70 is required to discharge nascent proteins for transport (6–10).

Evidence for participation of YDJ1p in intracellular events that require Hsp70SSA1 comes from observations that temperature-sensitive mutations in YDJ1p cause defects in protein transport into mitochondria (10) and the endoplasmic reticulum at the non-permissive temperature. These results support observations made here that YDJ1p and Hsp70SSA1 interact functionally. However, in addition to stimulating the ATPase activity of dnaKp, E. coli dnaJp can bind several protein substrates independent of dnaKp (1, 5, 24–26) and may actually target dnaKp to substrates by altering their conformation (3, 24, 25). Therefore, the possibility that YDJ1p acts as a chaperone independent of Hsp70SSA1 in protein trafficking events cannot be excluded by data presented here. However, YDJ1p does not form a complex with 125I-CMLA or other peptide substrates that is stable enough to withstand electrophoresis on native gels (Fig. 3A, lane 2). This is not surprising, since gel filtration experiments indicate that dnaJp does not bind to linear substrates such as CMLA but does bind proteins exhibiting tertiary structure such as folding intermediates of rhodanese (5). We are currently attempting to determine if YDJ1p binds to proteins competent for folding.

The stability of Hsp70SSA1–CMLA complexes observed here in the presence of ATP (Fig. 3, A and B) is noteworthy since experiments with the other Hsp70 homologs dnaKp, Hsc73, and BIP have shown that inclusion of ATP alone in reaction mixtures is sufficient to release the majority of bound substrate (5, 20–22). Tight binding of polypeptides in the presence of ATP may reflect a specialization of Hsp70SSA1 that allows for maintenance of unassembled or nascent proteins in an assembly or translocation competent form prior to release from the chaperone upon its interaction with YDJ1p. Indeed, there is precedent for specialization of Hsp70 function. In vitro assays for uncoating of clathrin vesicles (23) and lysosomal protein degradation (28) demonstrate that there are...
large differences in the activity of different Hsp70 homologs. In yeast, there is evidence for specialization of the different cytosolic dnaJ homologs as SIS1 and YDJ1 deletion strains exhibit different phenotypes (9-11). YDJ1p is farnesylated posttranslationally, whereas SIS1p is not (11, 16).

Results reported here demonstrate that YDJ1p stimulates release of polypeptide substrates from Hsp70\textsuperscript{SSA1} through stimulation of ATP hydrolysis (Fig. 3, A and B). This is in contrast to a recent report in which E. coli dnaJp was found to stabilize substrate binding to dnaKp (5). Langer \textit{et al.} (5) propose that dnaJp, which stimulates dnaKp ATPase activity about 2-fold (4), acts to stabilize dnaKp-polypeptide complexes by driving the conversion of ATP-dnaKp complexes to ADP-dnaKp-complexes, which have higher affinity for polypeptide substrates (20). Since grpEp was found to stimulate dissociation of dnaKp-dnaJp-polypeptide complexes (5), nucleotide exchange catalyzed by grpEp was presumed to promote polypeptide release by stimulating rounds of ATP hydrolysis (4). In preliminary comparisons of polypeptide binding to Hsp70\textsuperscript{SSA1} no increase in the level of stable complex formation was observed when ATP was substituted for ADP in binding reactions. Furthermore, addition of ATP and YDJ1p to reaction mixtures containing Hsp70\textsuperscript{SSA1} polypeptide complexes formed in the presence of ADP resulted in complex dissociation similar to that observed in Fig. 3B (not shown). This result indicates that ATP hydrolysis stimulated by YDJ1p, not nucleotide exchange, is limiting in the dissociation of polypeptides bound to Hsp70\textsuperscript{SSA1} molecules.

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