THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 267, No. 29, Issue of October 15, pp. 20927-20931, 1992
Printed in U.S.A.

Regulation of Hsp70 Function by a Eukaryotic DnaJ Homolog*

(Received for publication, July 23, 1992)

Douglas M. Cyr‡, Xiangyang Lu, and Michael G. Douglas§

From the Department of Biochemistry and Biophysics, University of North Carolina Medical School, Chapel Hill, North Carolina 27599-7260

We report that a purified cytoplasmic Hsp70 homolog from *Saccharomyces cerevisiae*, Hsp70^{SSA1}, exhibits a weak ATPase activity, which is stimulated by a purified eukaryotic dnaJp homolog (YDJ1p). Stable complex formation between Hsp70^{SSA1} and the permanently unfolded protein carboxymethylated α-lactalbumin (CMLA) was assayed by native gel electrophoresis. The affinity of Hsp70^{SSA1} for CMLA appeared to be regulated by YDJ1p. Significant reduction in both CMLA-Hsp70^{SSA1} complex formation and the release of CMLA pre-bound to Hsp70^{SSA1} was observed only in the presence of both YDJ1p and ATP. Thus, Hsp70^{SSA1} and YDJ1p interact functionally in the execution of Hsp70^{SSA1} chaperone activities in the eukaryotic cell.

Molecular chaperones of the Hsp70[1] 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 chaperonin groEL (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). YDJ1p is farnesylated at a C-terminal Caa*X* box (where a is an aliphatic amino acid and *X* is any residue), and this modification appears to mediate partitioning of YDJ1p between the cytosol and different intracellular membranes (9, 16). To test for interactions between the abundant eukaryotic Hsp70 family members and dnaJ homologs, YDJ1p and a cytosolic Hsp70 homolog of *S. cerevisiae*, Hsp70^{SSA1}, were purified. The influence of YDJ1p 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 YDJ1p—YDJ1p was overexpressed in *E. coli* strain BL21 (DE3) as described previously (16). Cells from a 200-ml culture were isolated, 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 YDJ1p was eluted with a 0–300 mM NaCl gradient. Peak fractions containing YDJ1p were pooled and then dialyzed against buffer B (5 mM potassium phosphate, pH 7.0, 10 mm DTT). YDJ1p 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 YDJ1p 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 (Matα, 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 Hsp70^{SSA1}. Cells were harvested at OD_{660} = 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 $MgCl_2$, 0.5 mM EDTA, 10 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 Hsp70^{SSA1p} ATPase Activity—Purified Hsp70^{SSA1} was in-

* This work was supported in part by National Institutes of Health Grant GM36537 (to M. G. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by Postdoctoral Fellowship NC-90-F4 from the American Heart Association.

§ To whom correspondence should be addressed.

[1] The abbreviations used are: Hsp70, heat shock protein(s) of the 70-kDa family; BIP, Hsp70 homolog found in the lumen of the endoplasmic reticulum; Hsc73, heat shock cognate protein of 73 kDa; DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid; CMLA, carboxymethylated α-lactalbumin; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate.

cubated in reaction mixtures containing 50 mM Hepes, pH 7.4, 50 mM NaCl, 10 mM DTT, 2 mM $MgCl_2$, and ATP (as indicated, [α-^{32}P] ATP, 7.0×10^2 to 1.0×10^4 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 Hsp70^{SSA1} ATPase activity by YDJ1p.

Gel Shift Assay for ^{125}I-CMLA Binding to Hsp70^{SSA1p}—Binding reactions were carried out at 30 °C for 20 min in 20-μl reaction mixtures composed of the following: Hsp70^{SSA1} (2.5–3.0 μM), 50 mM Hepes, pH 7.0, 50 mM NaCl, 10 mM DTT, 0.1 mM EDTA, 0.4% bovine serum albumin, and ^{125}I-CMLA (0.7 μM, 4.5×10^5 cpm/pM). The concentrations of indicated reagents were: YDJ1p (0–3.4 μM), 2 mM $MgCl_2$, 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 ^{125}I-CMLA migration was specific for Hsp70^{SSA1} as bovine serum albumin, YDJ1p, and enolase, respectively, at 5 μg/reaction mixture had no effect on CMLA migration. The Hsp70^{SSA1} and ^{125}I-CMLA concentrations used are in the linear range for complex formation. To calculate binding in percent of control bands on the gel corresponding to ^{125}I-CMLA and ^{125}I-CMLA-Hsp70^{SSA1} complexes were excised from the dried gel and assayed for ^{125}I by γ counting. Under control conditions, an experimental variation in the level of ^{125}I-CMLA-Hsp70^{SSA1} complex formation was observed, 10–30% of ^{125}I-CMLA added to reaction mixtures formed a complex with Hsp70^{SSA1}. 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 Iodination—^{125}I-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 (Hsp70^{SSA1}) 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* lack 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 Hsp70^{SSA1} and YDJ1p, the influence of YDJ1p on Hsp70^{SSA1} ATPase activity was determined. Hsp70^{SSA1} 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 Hsp70^{SSA1} ATPase activity approximately 10-fold at all time points tested (Fig. 2*A*). The -fold stimulation of Hsp70^{SSA1} ATPase activity by YDJ1p was constant over a range of ATP concentrations which were above and below the K_m of Hsp70^{SSA1} for ATP (approximately 2.5 μM). Thus, YDJ1p influences the maximal velocity of the ATPase reaction and not the affinity of Hsp70^{SSA1} for ATP (Fig. 2*B*). Maximal stimulation of Hsp70^{SSA1} ATPase activity was observed at a YDJ1p:Hsp70^{SSA1} molar ratio near 1.0 (Fig. 2*C*). Preincubation of YDJ1p for 10 min at 70 °C prior to assay reduced stimulation of Hsp70^{SSA1} 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.

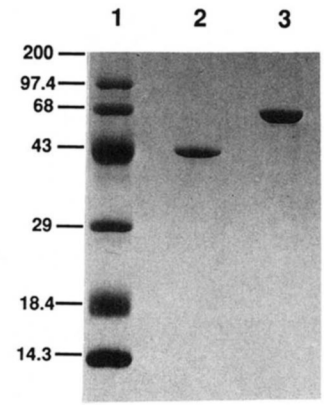

FIG. 1. **Analysis of purified YDJ1p and Hsp70^{SSA1} by SDS-PAGE.** Respective proteins (1.5 μg) were run on a 12.5% polyacrylamide mini-gel and the gel stained with Coomassie Brilliant Blue R-250. *Lane 1*, molecular mass markers (given in kDa); *lane 2*, YDJ1p; *lane 3*, Hsp70^{SSA1}.

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 Hsp70^{SSA1}, a gel shift assay was developed to monitor stable interactions between Hsp70^{SSA1} 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 ^{125}I and utilized as a substrate (5, 20). ^{125}I-CMLA migrated with high mobility on a native polyacrylamide gels (Fig. 3*A*, *lane 1*). Incubation with YDJ1p had no influence on ^{125}I-CMLA migration (Fig. 3*A*, *lane 1 versus 2*). However, incubation with Hsp70^{SSA1} shifted migration of ^{125}I-CMLA to a position coincident to that of Hsp70^{SSA1}, indicating the proteins form a stable complex (Fig. 3*A*, *lane 1 versus 3*). ^{125}I-CMLA-Hsp70 complex formation was essentially unchanged in the presence of ATP or YDJ1p alone (Fig. 3*A*, *lane 3 versus 4* and *6*). However, the combination ATP and YDJ1p reduced Hsp70^{SSA1}-CMLA complex formation by over 70%. When ATP was replaced by AMP-PNP, a non-hydrolyzable ATP analog, YDJ1p had no significant influence on ^{125}I-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 ^{125}I-CMLA from Hsp70^{SSA1,} binding assays were carried out in two steps. In the first step Hsp70^{SSA1} and ^{125}I-CMLA were incubated to allow complex formation. In the second step, the reaction mixture containing the ^{125}I-CMLA-Hsp70^{SSA1} complex was split and incubated further. Addition of ATP to the second reaction resulted in release of 20% of the CMLA bound to Hsp70^{SSA1p} in the first reaction (Fig. 3*B*, *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. 3*B*, *lane 1 versus 4*). Thus, the combination of YDJ1p and ATP not only prevents substrate binding to Hsp70^{SSA1} (Fig. 3*A*) but can also can 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 Hsp70^{SSA1}. When a peptide that is specifically recognized by the mitochondrial import apparatus (27), F1β 1–51, was employed as a substrate, stable complex formation with Hsp70^{SSA1} was

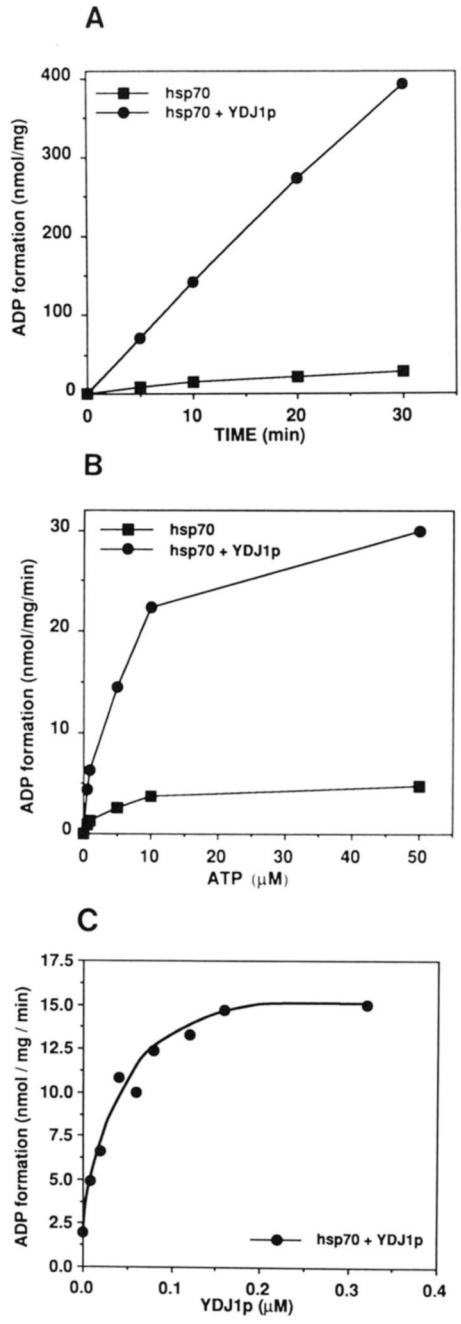

FIG. 2. **YDJ1p stimulates Hsp70^{SSA1} ATPase activity.** *A*, Kinetics of Hsp70^{SSA1} ATPase activity. Hsp70^{SSA1} (0.52 μM) and YDJ1p (0.67 μM), as indicated, were incubated with ATP (100 μM; 1.8×10^3 cpm/pM) in 60-μl reaction mixtures at 30 °C. At the specified time, duplicate 2-μl aliquots were removed and assayed for ADP formation. *B*, stimulation of Hsp70^{SSA1} ATPase activity by YDJ1p is independent of ATP concentration; Hsp70^{SSA1} (0.30 μM), YDJ1p (0.30 μM), and ATP (as indicated, 1.4×10^4 cpm/pM) were incubated in 20-μl reaction mixtures at 30 °C for 5 min. *C*, YDJ1p stimulates Hsp70^{SSA1} ATPase activity in a dose-dependent manner; Hsp70^{SSA1} (0.25 μM), ATP (50 μM, 7.0×10^2 cpm/pM), and YDJ1p (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. 3*A*) the combination of ATP and YDJ1p was required to effect significant substrate release,[2] indicating that YDJ1p regulates the interactions between Hsp70^{SSA1} and at least two different protein substrates.

ATP-dependent reduction of ^{125}I-CMLA-Hsp70 complex

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

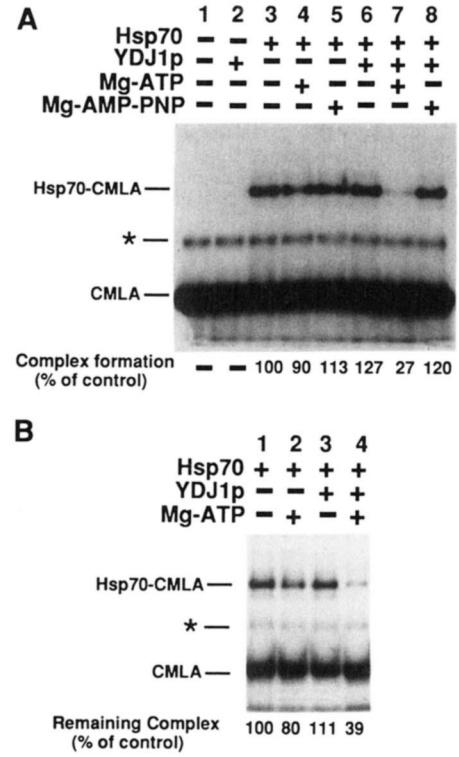

FIG. 3. **YDJ1p stimulates CMLA-Hsp70^{SSA1} complex dissociation.** *A*, gel shift analysis of ^{125}I-CMLA-Hsp70^{SSA1} complex formation. Hsp70^{SSA1} (2.5 μM) and ^{125}I-CMLA (0.7 μM, 4.5×10^5 cpm/pM) were incubated with the indicated additions at 30 °C for 20 min. Complex formation was analyzed by electrophoresis of reaction mixtures on 10–15% linear gradient native gels (see "Experimental Procedures" for details). *CMLA* denotes the migration of ^{125}I-CMLA in the absence of Hsp70. *Asterisk* (*) marks the migration of a radiolabeled contaminant in the ^{125}I-CMLA preparation. The intensity of the * band was constant under all assay conditions. Hsp70-CMLA denotes migration of ^{125}I-CMLA to a position on the gel coincident to that of Hsp70 as determined by staining gels with Coomassie Blue R-250 prior to autoradiography. 100% of control binding represents 10% of the total ^{125}I-CMLA added to reaction mixtures (*lane 3*). *B*, YDJ1p stimulates polypeptide release from Hsp70^{SSA1}. For this experiment reactions were carried out in a two-step process. First, Hsp70 (3.0 μM) and ^{125}I-CMLA (0.4 μM, 4.5×10^5 cpm/pM) were incubated in an 80-μl reaction mixture at 30 °C for 20 min to allow ^{125}I-CMLA-Hsp70^{SSA1} complex formation. Aliquots (15 μl) of the initial reaction mixture were then removed and incubated in a second 20-μl reaction mixture, with indicated additions, for 20 min at 30 °C and then analyzed for complex formation as described above. Control assays show that the level of ^{125}I-CMLA -Hsp70^{SSA1} complex in Fig. 3*B* (*lane 1*) is the same as that observed when complex formation is assayed immediately after the first incubation. Therefore changes in the levels of complex observed are resultant from a shift in the equlibrium of the binding reaction in response to addition of ATP or the combination of ATP and YDJ1p. 100% of control binding represents 29% of the total ^{125}I-CMLA added to reaction mixtures For details pertaining to the composition of reaction mixtures see "Experimental Procedures."

formation by YDJ1p was, however, never complete. The small amount of ^{125}I-CMLA that remained bound might be due to either the formation of a nonspecific complex or due to a specific association in which the Hsp70^{SSA1} molecule could either not respond to YDJ1p or which may require an additional component for complete release. To establish the specificity of ^{125}I-CMLA-Hsp70^{SSA1} complex formation, unlabeled competitor CMLA was included in reactions to determine if the level of CMLA bound to Hsp70^{SSA1} could be reduced below that measured in the presence of YDJ1p and ATP (Fig. 4*A*, *lane 2*). At a 100-fold molar excess of unlabeled CMLA, the Hsp70^{SSA1}-CMLA complex was reduced to only 22% of control valves. This was the same level of residual binding observed

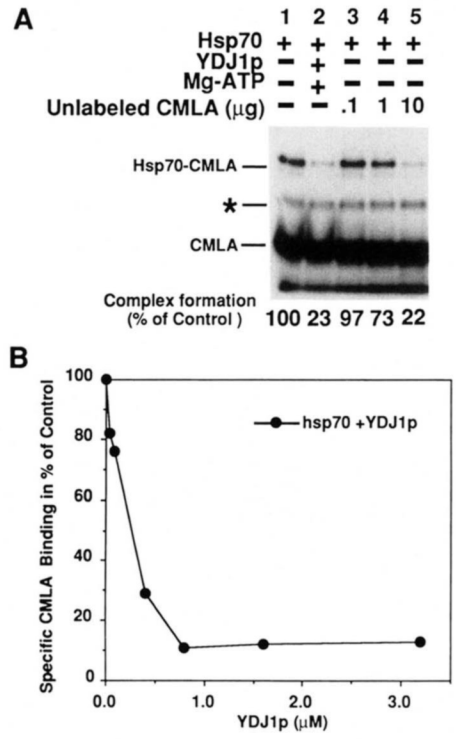

FIG. 4. **Specificity of ^{125}I-CMLA binding to Hsp70^{SSA1}.** *A*, unlabeled CMLA competes for binding of ^{125}I-CMLA to Hsp70^{SSA1}. Hsp70^{SSA1} (2.5 μM), ^{125}I-CMLA (0.35 μM, 4.5 × 10^5 cpm/pM), and unlabeled CMLA (as indicated) were incubated at 30 °C for 20 min. Assay for binding of ^{125}I-CMLA to Hsp70^{SSA1} was carried out as described in the legend to Fig. 3*A*. ^{125}I-CMLA and unlabeled CMLA behave identically on native gels. CMLA at 10 μg/reaction mixture was 33 μM. Other reagent concentrations were: YDJ1p (2.45 μM), 2 mM $MgCl_2$, and 1 mM ATP. 100% of control binding (*lane 1*) represents 12% of total label added to reactions. *B*, YDJ1p-dependent reduction of specific ^{125}I-CMLA binding to Hsp 70^{SSA1}. Shown is quantitation of a binding experiment carried out as described in the legend to Fig. 3*A*. Reaction mixtures containing Hsp70^{SSA1} (2.75 μM), ^{125}I-CMLA (0.35 μM, 4.5 × 10^5 cpm/pM), 2 mM $MgCl_2$, 1 mM ATP, and YDJ1p (as indicated) were incubated for 20 min at 30 °C. Specific binding was determined by subtracting binding of ^{125}I-CMLA which could not be competed by addition of 10 μg of unlabeled CMLA to reaction mixtures from total binding observed. Total binding and nonspecific binding represented 20 and 4% of ^{125}I-CMLA, respectively.

the presence of YDJ1p and ATP (Fig. 4*A*, *lane 2 versus 5*). This indicates that the Hsp70^{SSA1}-CMLA complex, which was resistant to YDJ1p and ATP, results from nonspecific interactions.

To correlate stimulation of Hsp70^{SSA1} ATPase activity by YDJ1p (Fig. 2) with decreases in specific substrate binding to Hsp70^{SSA1}, the ratio of YDJ1p to Hsp70^{SSA1} required to maximally reduce ^{125}I-CMLA-Hsp70^{SSA1} complex formation was determined (Fig. 4*B*). When the nonspecific association of ^{125}I-CMLA to Hsp70^{SSA1} was subtracted, YDJ1p and ATP reduced specific substrate binding by 90% at a YDJ1p: Hsp70^{SSA1} molar ratio similar to that observed for maximal stimulation of ATP hydrolysis by YDJ1p (Fig. 4*B*).

DISCUSSION

Data presented here provide the first evidence for modulation of the activity of an eukaryotic Hsp70 family member by a regulatory factor. Since the ATPase activity of some Hsp70 homologs can be stimulated by interaction with polypeptide substrates (1, 21) the possibility that YDJ1p acts at an allosteric site on Hsp70^{SSA1} to regulate its function or the polypeptide binding site needed to be resolved. Data presented here argue that YDJ1p is not acting as a substrate of Hsp70^{SSA1} but as a regulator. 1) Stoichiometry data (Figs. 1*C* and 4*B*) indicate that equamolar YDJ1p concentrations are required to stimulate ATP hydrolysis maximally and also to reduce polypeptide-Hsp70^{SSA1} complex formation. 2) YDJ1p-dependent reduction of Hsp70-CMLA complex formation requires ATP hydrolysis (Fig. 3*A*). 3) YDJ1p stimulates ATP-dependent release of substrates pre-bound to Hsp70^{SSA1}. 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 Hsp70^{SSA1} ATPase activity maximally.[2] Furthermore, interactions between YDJ1p and Hsp70^{SSA1} do not appear as stable as those between polypeptide substrates and Hsp70^{SSA1} since no alteration in the mobility of ^{125}I-CMLA-Hsp70^{SSA1} complex is observed on native gels upon addition of YDJ1p to reaction mixtures (Fig. 3*A*, *lane 3 versus 6*).

The lack of stable YDJ1p binding to Hsp70^{SSA1} 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 Hsp70^{SSA1} (Fig. 3*B*) 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 Hsp70^{SSA1} comes from observations that temperature-sensitive mutations in YDJ1p cause defects in protein transport into mitochondria (10)[3] and the endoplasmic reticulum[3] at the non-permissive temperature. These results support observations made here that YDJ1p and Hsp70^{SSA1} 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 Hsp70^{SSA1} in protein trafficking events cannot be excluded by data presented here. However, YDJ1p does not form a complex with ^{125}I-CMLA or other peptide substrates that is stable enough to withstand electrophoresis on native gels (Fig. 3*A*, *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 Hsp70^{SSA1}-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 Hsp70^{SSA1} 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

[3] Caplan, A. J., Cyr, D. M., and Douglas, M. G. (1992) *Cell*, in press.

large differences in the activity of different Hsp70 homologs. In yeast, there is evidence for specialization of the different cytosolic dnaJp 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 $Hsp70^{SSA1}$ 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 *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 $Hsp70^{SSA1}$ no increase in the level of stable complex formation was observed when ADP was substituted for ATP in binding reactions. Furthermore, addition of ATP and YDJ1p to reaction mixtures containing $Hsp70^{SSA1}$-polypeptide complexes formed in the presence of ADP resulted in complex dissociation similar to that observed in Fig. 3*B* (not shown). This result indicates that ATP hydrolysis stimulated by YDJ1p, not nucleotide exchange, is limiting in the dissociation of polypeptides bound to $Hsp70^{SSA1}$ molecules.

Acknowledgments—We thank Elizabeth Craig for providing yeast strain MW141, Avrom Caplan for providing the YDJ1 overexpressor strain, Ulrich Hartl for providing purified dnaK protein, Gregory Flynn for providing purified BIP protein. We also thank Avrom Caplan, Ken Kassenbrock, and David Nelson for helpful discussions throughout the course of this work.

REFERENCES

1. Gething, M. J., and Sambrook, J. (1992) *Nature* **355,** 33–45
2. Craig, E. A., and Gross, C. A. (1991) *Trends Biochem. Sci.* **16,** 135–140
3. Ang, D., Liberek, K., Skowyra, D., Zylicz, M., and Georgopoulos, C. (1991) *J. Biol. Chem.* **266,** 24233–24236
4. Liberek, K., Marszalek, J., Ang, D., Georgopoulos, L., and Zylicz, M. (1991) *Proc. Nat. Acad. Sci. U. S. A.* **8,** 2874–2878
5. Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M. K., and Hartl, F.-U. (1992) *Nature* **356,** 683–689
6. Deshaies, R., Koch, B., Werner-Washburne, M., Craig, E., and Schekman, R. (1988) *Nature* **332,** 800–805
7. Chirico, W. J., Waters, M. G., and Blobel, G. (1988) *Nature* **332,** 805–810
8. Shi, Y., and Thomas, J. O. (1992) *Mol. Cell. Biol.* **12,** 2186–2192
9. Caplan, A., and Douglas, M. G. (1991) *J. Cell Biol.* **114,** 609–621
10. Atencio, D. P., and Yaffe, M. P. (1992) *Mol. Cell. Biol.* **12,** 283–291
11. Luke, M., Sutton, A., and Arndt, K. (1991) *J. Cell Biol.* **114,** 623–638
12. Rothblatt, J. A., Deshaies, R. J., Sanders, S. L., Daum, G., and Schekman, R. (1989) *J. Cell Biol.* **109,** 2641–2652
13. Sadler, I., Chiang, A., Kurihara, T., Rothblatt, J., Way, J., and Silver, P. (1989) *J. Cell Biol.* **109,** 2665–2675
14. Blumberg, H., and Silver, P. (1991) *Nature* **349,** 327–330
15. Bork, P., Sander, C., and Valencia, A. (1992) *Trends Biochem. Sci.* **17,** 29
16. Caplan, A., Tsai, J., Casey, P., and Douglas, M. G. (1992) *J. Biol. Chem.* **267,** 18890–18895
17. Welch, W. J., and Feremisco, J. R. (1985) *Mol. Cell. Biol.* **5,** 1229–1237
18. Kassenbrock, C. K., and Kelly, R. B. (1989) *EMBO J.* **8,** 1461–1467
19. Sholomai, J., and Kornberg, A. (1980) *J. Biol. Chem.* **255,** 6789–6793
20. Palleros, D., Welch, W., and Fink, A. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 5719–5723
21. Flynn, G., Chappell, T., and Rothman, J. (1989) *Science* **245,** 385–390
22. Lieberek, K., Skowyra, D., Zylicz, M., Johnson, C., and Georgopoulos, L. (1991) *J. Biol. Chem.* **266,** 14491–14496
23. Gao, B., Biosca, J., Craig, E. A., Greene, L. E., and Eisenberg, E. (1991) *J. Biol. Chem.* **266,** 19565–19571
24. Wickner, S., Hoskins, J., and McKenny, K. (1991) *Nature* **350,** 165–167
25. Georgopoulos, C., Ang, D., Liberek, K., and Zylicz, M. (1990) *Stress Proteins in Biology and Medicine,* pp. 1912–221, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26. Gamer, J., Bujard, H., and Bukau, B. (1992) *Cell* **69,** 833–842
27. Cyr, D. M., and Douglas M. G. (1991) *J. Biol. Chem.* **266,** 21700–21708
28. Terlecky, S. R., Chaing, H.-L., Olson, T. S., and Dice, J. F. (1992) *J. Biol. Chem.* **267,** 9202–9209