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Differential Regulation of Hsp70 Subfamilies by the Eukaryotic DnaJ Homologue YDJ1*

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In *Saccharomyces cerevisiae* Ydj1p, a DnaJ homolog, is localized to the cytosol with the Ssa and Ssb Hsp70 proteins. Ydj1p helps facilitate polypeptide translocation across mitochondrial and endoplasmic reticulum membranes (Caplan, A. J., Cyr, D. M., and Douglas, M. G. (1992) *Cell* 71, 1143–1155) and can directly interact with Ssa1p to regulate chaperone activity (Cyr, D. M., Lu, X., and Douglas, M. G. (1992) *J. Biol. Chem.* 267, 20927–20931). In this study, the role of Ydj1p in modulating ATP-dependent reactions catalyzed by Ssa and Ssb Hsp70 proteins has been examined using purified components and compared with that of other Hsp70 homologs BiP and DnaK. Ssa1p, Ssa2p, and Ssb1/2p all formed stable complexes with the mitochondrial presequence peptide, F1β(1–51). ATP alone had only modest effects on polypeptide complex formation with Ssa1p and Ssa2p, but prevented the majority of polypeptide binding to BiP and DnaK. ATP by itself also reduced polypeptide binding to Ssb1/2p to a level that was intermediate between that observed for the Ssa Hsp70 proteins tested and BiP and DnaK. ATP hydrolysis by Ssa1p, Ssa2p, and Ssb1/2p occurred at similar rates. Ydj1p was a potent modulator of the both the ATPase and polypeptide binding activities of Ssa1p and Ssa2p. In contrast, Ydj1p had little effect on the ATPase and polypeptide binding activity of Ssb1/2p. Therefore the chaperone-related activities of Ssa and Ssb Hsp70 proteins exhibit significant differences in sensitivity to ATP and YDJ1p. These data indicate that regulation of Hsp70 activity by DnaJ homologs can be specific. The specificity of interactions between Ydj1p and the Ssa and Ssb Hsp70 proteins observed could contribute in determining the functional specificity of these chaperones in the cytosol. In related experiments, F1β(1–51) was found to reduce the extent to which Ydj1p stimulated Ssa1p ATPase activity. This effect correlated with the formation of F1β(1–51)·Ssa1p complexes. We propose that intramolecular communication between the polypeptide binding, ATPase and DnaJ regulatory domains on Ssa1p plays a role in the regulation of chaperone activity.

Hsp70 proteins play an essential role in cellular physiology by acting as molecular chaperones to facilitate the biogenesis and degradation of proteins (reviewed by Gething and Sambrook (1992) and Hendrick and Hartl (1993)). The key to chaperone function is the ability of Hsp70 proteins to transiently bind polypeptides and release them in an ATP-dependent manner (Pelham, 1990). In *Saccharomyces cerevisiae* five gene families (SSA1–4, SSB1–2, SSC1, SSD1/KAR2, and MSI3) encode nine different Hsp70 homologs (Craig *et al.*, 1993; Shirayma *et al.*, 1993). Ssc1p and Kar2p are localized in the mitochondrial matrix and endoplasmic reticulum lumen, respectively, and both help facilitate protein import into the organelles in which they reside (Kang *et al.*, 1990; Manning-Krieg *et al.*, 1991; Nugyen *et al.*, 1991; Sanders *et al.*, 1992). Both the Ssa and Ssb Hsp70 proteins are localized to the cytosol, but they appear to have different primary functions. The SSA Hsp70 gene family is essential for viability (Werner-Washburne *et al.*, 1987). Ssa1p and Ssa2p, which exhibit about 98% sequence identity to each other (Slater and Craig, 1989), help maintain preproteins in conformations competent for translocation across subcellular membranes (Deshaies *et al.*, 1988; Chirico *et al.*, 1988). On the other hand, Ssb1p and Ssb2p, which exhibit about 99% sequence identity to each other, are only essential for growth at low temperatures (Craig and Jacobsen, 1985; Craig *et al.*, 1993). The primary function of the Ssb Hsp70 proteins appears be in facilitating the transit of nascent polypeptide chains through the large subunit of ribosomes during protein synthesis (Nelson *et al.*, 1992).

The functional differences observed for the Ssa and Ssb Hsp70 proteins are likely to result from divergent evolution of the different gene families, Ssa1p and Ssa2p exhibit only 60% sequence identity to Ssb1p and Ssb2p (Craig *et al.*, 1993). However, the mechanisms that determine the specificity of Ssa and Ssb Hsp70 protein function are not clear. The unique amino acid compositions of the Ssa and Ssb Hsp70 appear to confer differences in the specificity of polypeptide binding by these proteins (Gao *et al.*, 1991). Additionally, polypeptide release form Ssa1p appears to be less sensitive to ATP than that observed for other Hsp70 homologs (Cyr *et al.*, 1992). Another factor that may contribute to determining functional specificity is regulation of Hsp70 activity by cytosolic DnaJ homologs. In *Escherichia coli*, biochemical and genetic evidence indicates that DnaJ together with GrpE synergistically regulate the activity of Hsp70 (DnaK); DnaJ regulates rates of ATP hydrolysis and GrpE stimulates ADP/ATP exchange (Georgopolous *et al.*, 1990; Liberek *et al.*, 1991). *S. cerevisiae* has at least two DnaJ homologs that are localized to the cytosol; Sis1p and the more abundant Ydj1p (Caplan and Douglas, 1991; Atencio and Yaffe, 1992; Luke *et al.*, 1991). Like all DnaJ homologs both SiS1p and Ydj1p contain a highly conserved motif termed the "J domain," which is thought to interact with Hsp70 (Silver and Way, 1993). The functions of Sis1p and Ydj1p appear to be specialized. SIS1 can suppress the severe growth defects observed in YDJ1 null strains at 30 °C, but YDJ1 cannot suppress the loss of viability observed in SIS1 null strains (Caplan and Douglas, 1991; Luke

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et al., 1991). Sis1p is found in association with cytosolic ribosomes and plays a role in translation initiation (Zhong and Arndt, 1993). Ydj1p is farnesylated and distributed between intracellular membranes and the cytosol (Caplan and Douglas, 1991; Caplan *et al.*, 1992b). Ydj1p participates in pathways that require Ssa1p and Ssa2p in the cell; temperature-sensitive mutations in YDJ1 cause defects in protein translocation into both mitochondria and the endoplasmic reticulum (Caplan *et al.*, 1992a; Atencio and Yaffe, 1992). Purified Ydj1p can directly interact with Ssa1p to regulate its ATPase and polypeptide binding activity (Cyr *et al.*, 1992).

The specificity of interactions between Ydj1p and cytosolic Hsp70 homologs other than Ssa1p is unknown. Purified components have been used to compare the influence of ATP and Ydj1p on the chaperone-related activities of Ssa1p, Ssa2p, and Ssb1/2p. We find YDJ1p to be a strong modulator of Ssa1p and Ssa2p ATPase and polypeptide binding activities. However, Ydj1p had extremely weak effects on the ATPase and polypeptide binding activity of Ssb1/2p. The polypeptide binding activity of Ssa1p and Ssa2p also appeared to be less sensitive to ATP than that of other Hsp70 proteins tested. These results indicate that differential sensitivity of polypeptide binding to ATP and YDJ1p could play a role in determining the functional specificity of the Ssa and Ssb Hsp70 proteins in the cytosol.

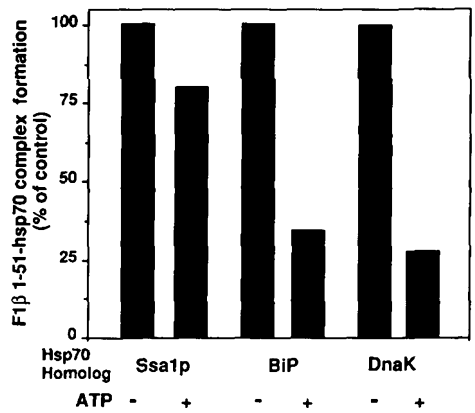

FIG. 1. **ATP has differential effects on complex formation between F1β(1–51) and different Hsp70 homologs.** Ssa1p, BiP, and DnaK (2.5 μM) were incubated with ^{125}I-F1β(1–51) (0.4 μM, 9.6 × 10^3 cpm/pM) for 20 min at 30 °C. Reaction mixtures were then assayed for complex formation by electrophoresis on a 7.5–15% linear gradient native gel. Displayed is quantitation of a representative experiment as percentage of control. 100% binding to Ssa1p, BiP, and DnaK in the absence of ATP was 13, 18, and 8% of the total ^{125}I-F1β(1–51) added to reaction mixtures, respectively. The ^{125}I-F1β(1–51) concentration used is in the linear range for binding to the respective Hsp70 homologs.

MATERIALS AND METHODS

Protein Purification—Ydj1p was overexpressed in *E. coli* and purified as previously described (Cyr *et al.*, 1992). Ssa1p was purified from yeast strain MW141 by ATP-agarose chromatography as previously described (Cyr *et al.*, 1992). Purified BiP was a gift from Dr. Gregory Flynn (University of Oregon). Purified DnaK was a gift from Dr. Frans-Ulrich Hartl (Sloan Kettering Institute, New York, NY). Ssa2p and Ssb1/2p were purified from yeast strain MW146 which is genetically engineered to express only Ssa2p and Ssb1/2p in the cytosol under normal growth conditions (Craig and Jacobsen, 1984). MW146 cells, 50 g wet weight, were suspended in 50 ml of 500 mM NaCl, 50 mM Hepes (pH 7.4), 10 mM DTT,[1] 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, eight 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) resins as described previously (Cyr *et al.*, 1992). Pooled Hsp70 fractions contained two bands corresponding to Ssa2p and Ssb1/2p (Fig. 3, *lane 3*) (Gao *et al.*, 1991). To resolve Ssa2p and Ssb1/2p from each other, 5 mg of the mixture was loaded onto a 1 × 4-cm hydroxyapatite column (Bio-Rad) equilibrated with 10 mM potassium phosphate buffer, pH 7.2, made 10 mM in DTT and 0.5 mM in phenylmethylsulfonyl fluoride. The column was washed with five volumes of equilibration buffer. Proteins were eluted with a 50-ml 10–400 mM potassium phosphate gradient. Elution of both Hsp70 homologs peaked at approximately 150 mM potassium phosphate. Fortuitously, Ssa2p eluted alone prior to the peak at 80–120 mM potassium phosphate. Ssb1/2p eluted in relatively pure form after the peak at 190–250 mM potassium phosphate buffer. Shown in *lane 2* of Fig. 3 is the pooled Ssa2p eluted prior to the peak and in *lane 5* is the pooled Ssb1/2p which eluted after the peak. Respective pooled fractions were dialyzed against 10 mM Hepes, pH 7.0, 50 mM NaCl, 10 mM DTT, and 10% glycerol), concentrated to 2.5 mg/ml, 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.

Assay of Hsp70 ATPase Activity—Purified Hsp70 homologs were incubated in reaction mixtures containing 10 mM Hepes, pH 7.4, 20 mM NaCl, 10 mM DTT, 1 mM $MgCl_2$, and ATP (as indicated, [α-^{32}P]ATP, 1 × 10^3 to 2.0 × 10^5 cpm/pM). Reaction mixtures were set up on ice, started by a shift to 30 °C, and incubated for 5 min. Reactions were stopped by placing them back on ice, and duplicate 2-μl aliquots were assayed for ADP formation by thin layer chromatography on polyethyleneimine-cellulose plates (Cyr *et al.*, 1992). Spontaneous ADP formation was also assayed and subtracted prior to calculation for rates of ATP hydrolysis. The kinetics of Hsp70 ATPase activity are linear for at least 20 min under these experimental conditions.

Gel Shift Assay for ^{125}I-Polypeptide binding to Hsp70—Reduced α-carboxymethylated lactalbumin (Sigma) and F1β(1–51) (Cyr and Douglas, 1991) were iodinated by labeling 100 μg of respective polypeptide with 0.5 mCi of carrier-free NaI (ICN) using Iodo-Gen (Pierce) as described by the manufacturer. Binding reactions were carried out at 30 °C for 20 min in 20-μl reaction mixtures composed of the following: Hsp70 (as indicated), 50 mM Hepes, pH 7.0, 20 mM NaCl, 10 mM DTT, 0.1 mM EDTA, 0.4% bovine serum albumin, and ^{125}I-substrate (as indicated). When present other reagent concentrations were as follows: $MgCl_2$, 2 mM; ATP, 1 mM; AMP-PNP, 1 mM. After incubation, reaction mixtures were diluted 2-fold with ice-cold 2 × reaction buffer made 20% (v/v) in glycerol and 0.01% in bromphenol blue. Diluted samples were loaded directly onto linear gradient native gels and run on ice at 10 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-substrates was specific for Hsp70 homologs, as bovine serum albumin, Ydj1p, and enolase, respectively, at 5 μg/reaction had no effect on migration. Complex formation between CMLA and F1β(1–51) and the respective Hsp70 homologs was maximal at the 20-min time point, indicating the binding reaction reached equilibrium during the incubation period. The Hsp70 homolog and ^{125}I-substrate concentrations used are in the linear range for complex formation. To calculate binding as a percentage of control, bands on the gel corresponding ^{125}I-substrate-Hsp70 complexes were excised from the dried gel and assayed for ^{125}I by γ counting. Depending on the experiment ^{125}I-substrate-Hsp70 complex formation accounted for 8–18% of total substrate added to reaction mixtures. This level of complex formation has previously been documented in gel filtration assays that monitor complex formation between Hsp70 homologs and unfolded substrates (Langer *et al.*, 1992; Palleros *et al.*, 1991). The specificity of substrate binding to Hsp70 homologs in this assay system was previously described (Cyr *et al.*, 1992).

RESULTS

Characterization of Complex Formation between F1β(1–51) and Ssa1p—In our previous study we observed that CMLA·Ssa1p complexes were stable in the presence of ATP (Cyr *et al.*, 1993). To follow up this observation, the influence of ATP on binding of the peptide F1β(1–51) by Ssa1p, BiP, and DnaK was examined (Fig. 1). F1β(1–51) is a mitochondrial presequence-containing peptide that represents amino acids 1–51 of the F1-ATPase β subunit precursor protein. Prior characterization shows that F1β(1–51) is specifically recognized by the mitochondrial import apparatus and imported into mito-

[1] The abbreviations used are: DTT, dithiothreitol; AMP-PNP, adenyl-5′-yl imidodiphosphate.

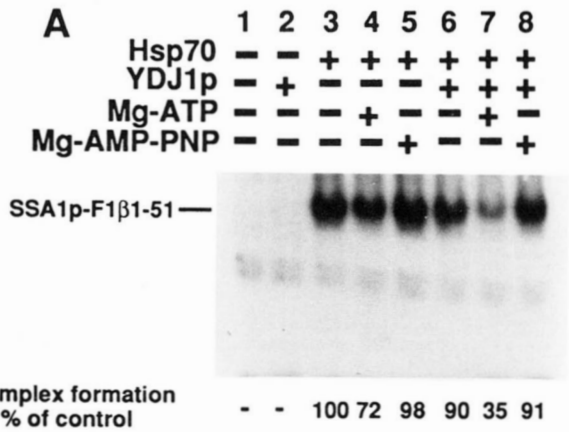

FIG. 2. **F1β(1–51) and CMLA form complexes with Ssa1p in the presence of ATP.** *A*, gel-shift analysis of ^{125}I-F1β(1–51)-Ssa1p complex formation. Ssa1p (2.5 μM) and ^{125}I-F1β(1–51) (0.4 μM, 9.6×10^3 cpm/pM), along with the indicated additions, were incubated in gel shift buffer for 20 min at 30 °C. Complex formation was analyzed by electrophoresis of reaction mixtures on 10–15% linear gradient native gels (see "Materials and Methods" for details). Ssa1p-F1β(1–51) denotes the migration of ^{125}I-F1β(1–51) to a position on the gel coincident to that of Hsp70 as determined by staining gels with Coomassie Brilliant Blue R-250 prior to autoradiography. 100% of control binding represents 11% of the total ^{125}I-F1β(1–51) present in reaction mixtures (*lane 3*). *B*, comparison of CMLA and F1β(1–51) binding to Ssa1p. The *bar graphs* represent the average quantitation of four individual binding experiments ± standard error. Experiments were carried out as described above. When present ^{125}I-CMLA was 0.7 μM (4.4 × 5 cpm/pM). On average, 12% of the respective radiolabeled substrates were found in association with Ssa1p under control conditions. Cold CMLA and F1β(1–51) indicate the presence a 100-fold molar excess of the respective non-labeled substrates in reaction mixtures.

chondria (Cyr and Douglas, 1991; Hoyt *et al.*, 1991).[2] Ssa1p, BiP, and DnaK all formed stable complexes with ^{125}I-labeled F1β(1–51) (Fig. 1). When saturating concentrations of ATP were added to reaction mixtures, complex formation between F1β(1–51) and the three different Hsp70 homologs was reduced (Fig. 1). Interestingly, ATP reduced complex formation between F1β(1–51) and both BiP and DnaK by 70%, but reduced complex formation with Ssa1p by only 20% (Fig. 1). At F1β(1–51)

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

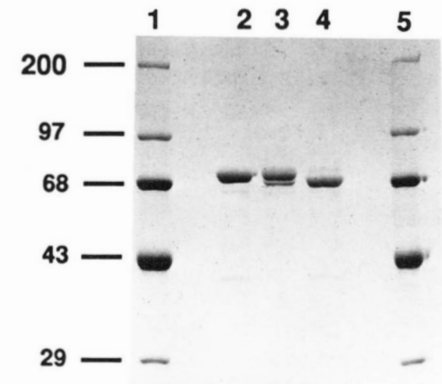

FIG. 3. **SDS-PAGE analysis of the purified *S. cerevisiae* Hsp70 homologs Ssa2p and Ssb1/2p.** Respective samples were analyzed on a 12.5% polyacrylamide minigel. Proteins were stained with Coomassie Brilliant Blue R-250. *Lanes 1* and *5*, molecular weight markers (weights are given in kDa). *Lane 2*, 1.5 μg of purified Ssa2p. *Lane 3*, 1.5 μg of a mixture of Ssa2p, the upper band, and Ssb1/2p, the lower band. 73 and 26% of total protein in *lane 3* was accounted for by Ssa2p and Ssb1/2p bands, respectively. *Lane 4*, 1.5 μg of purified Ssb1/2p. Ssa2p and Ssb1/2p proteins were resolved from the Ssa2/Ssb1/2p protein mixture shown in *lane 3* by hydroxyapatite chromatography. The Ssa2/Ssb1/2p mixture was purified from *S. cerevisiae* strain MW146 (see "Materials and Methods" for details). Ssa2 and Ssb1/2p shown in *lanes 2* and *3* were greater than 95% pure as determined by scanning laser densitometry. The Ssb1/2p fraction contains less than 1.5% Ssa2p as a contaminant. The minor 39-kDa contaminant in *lanes 2* and *4* appears as a result from slight degradation of Ssa2p and Ssb1/2p during hydroxyapatite chromatography as this material was not present in the mixture of these proteins (*lane 3*).

concentrations above and below saturation for complex formation, ATP did not reduce complex formation with Ssa1p by more than 30% (not shown). Ssa1p, BiP, and DnaK hydrolyzed ATP at similar rates in the presence and absence of F1β(1–51) (not shown). Thus, the rates of ATP hydrolysis by the different Hsp70 homologs do not appear to give rise to the differences observed. This comparison provides evidence that polypeptide binding to different Hsp70 proteins exhibits differential sensitivity to ATP.

The ability of Ydj1p to modulate levels of complex formation between Ssa1p and F1β(1–51) was examined next (Fig. 2). Little F1β(1–51) migrated into native gels when the peptide was incubated alone or with Ydj1p (Fig. 2*A*, *lanes 1* and *2*). However, after incubation with Ssa1p, F1β(1–51) migrated into the gel to a position coincident to that of Ssa1p (Fig. 2*A*, *lane 3*). Similar to Fig. 1, inclusion of ATP in incubations reduced complex formation around 25% (Fig. 2*A*, *lane 4*), while inclusion of AMP-PNP, Ydj1p, or the combination of Ydj1p and AMP-PNP had no effect (Fig. 2*A*, *lanes 5*, *6*, and *8*). The combination of Ydj1p and ATP reduced complex formation by 65% (Fig. 2*A*, *lane 7*). When compared side by side, F1β(1–51) binding to Ssa1p is similar to that of CMLA (Fig. 2*B*). On average ATP alone has no effect on CMLA binding to Ssa1p (Fig. 2*B*, *lane 1 versus lane 2*), whereas it reduces F1β(1–51) binding an average of 25% (Fig. 2*B*, *lane 5 versus lane 6*). Thus, experiments with two different substrates indicate that complex formation between Ssa1p and polypeptides has a lower sensitivity to ATP alone than other Hsp70 proteins. This difference apparently necessitates the need for Ydj1p to modulate the ATP-dependent release of the polypeptide substrates tested from Ssa1p.

Interactions between Ydj1p and the Ssa and Ssb Hsp70 Proteins—To examine the specificity of Ydj1p-Hsp70 interactions within the yeast cytosol, Ssa2p and Ssb1/2p were purified from *S. cerevisiae* strain MW146 (Craig and Jacobsen, 1984). MW146 is engineered to express only Ssa2p and Ssb1/2p under normal growth conditions in the cytosol (Craig and Jacobsen, 1984). Chromatography of MW146 post-ribosomal superna-

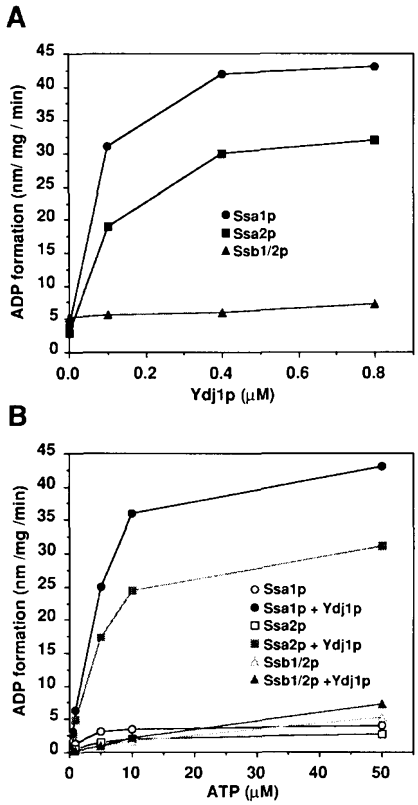

FIG. 4. **Ydj1p strongly stimulates the ATPase activity of Ssa1p and Ssa2p, but not Ssb1/2p.** *A*, respective Hsp70 homologs (0.52 μM), [α-^{32}P]-ATP (50 μM, 1.23 × 10^4 cpm/pM) and Ydj1p (as indicated) were incubated in 20 μl of ATPase buffer for 5 min at 30 °C. Reaction mixtures were then placed on ice, and duplicate 2-μl aliquots were removed and assayed for ADP formation. *B*, Ydj1p stimulates ATPase activity of the different Hsp70 homologs independent of ATP concentration. Respective Hsp70 homologs (0.31 μM), Ydj1p (0.43 μM), and ATP (as indicated, 2.1 × 10^5 cpm/pM) were incubated in 20-μl reaction mixtures at 30 °C for 5 min. For details pertaining to the composition of ATPase buffer and assay of ADP formation, see "Materials and Methods."

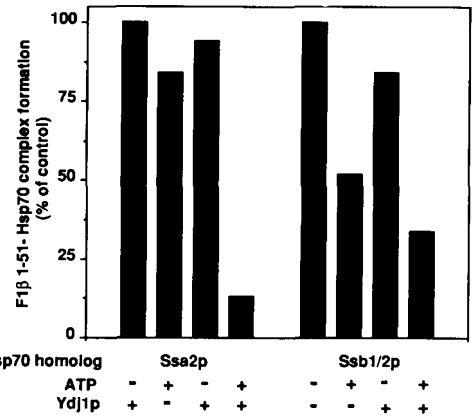

FIG. 5. **The influence of ATP and Ydj1p on ^{125}I-F1β(1–51) binding to Ssa2p and Ssb1/2p.** Respective Hsp70s (2.5 μM) and ^{125}I-F1β(1–51) (0.4 μM, 9.6 × 10^3 cpm/pM), along with the indicated additions, were incubated in gel shift buffer for 20 min at 30 °C. Complex formation was analyzed by electrophoresis of reaction mixtures on 5–15% linear gradient native gels (see "Materials and Methods" for details). Quantitation of this experiment is given as percentage of control. 100% of total binding to Ssa2p and Ssb1/2p represented 11 and 7.5% of the total ^{125}I-F1β(1–51) added to the respective reaction mixtures.

tants on ATP-agarose and DE52-cellulose yields a protein fraction that is essentially free of other proteins except for Ssa2p and Ssb1/2p (Fig. 3, *lane 3*). Ssb1/2p consists of Ssb1p and Ssb2p, which are 99% identical and are thus treated as the same protein (Craig *et al.*, 1993). Two-dimensional electrophoresis of similar protein fractions assigns Ssa2p as the upper band and Ssb1/2p as the lower band in the protein mixture (Fig. 3, *lane 3*) (Gao *et al.*, 1991). Ssa2p and the Ssb1/2p were resolved from each other by hydroxyapatite chromatography (see "Materials and Methods"). After this chromatography step, pooled Ssa2p fractions were essentially free of Ssb1/2p (Fig. 3, *lane 2*), while the pooled Ssb1/2p fraction contained a trace (less than 1.5%) of Ssa2p (Fig. 3, *lane 4*).

The influence of Ydj1p on the ATPase activity of Ssa1p, Ssa2p, and Ssb1/2p was determined next. The preparations of Ssa1p, Ssa2p, and Ssb1/2p used in this study hydrolyzed ATP at respective average rates of 3.4, 2.2, and 4.2 nmol/mg protein/min (not shown). Titration of Ydj1p into reaction mixtures increased the ATPase activity of Ssa1p and Ssa2p 10–12 fold at approximately equimolar concentrations with the respective Hsp70 homolog (Fig. 4*A*). However, a less than 1.5-fold increase in the ATPase activity of Ssb1/2p was observed under identical reaction conditions (Fig. 4*A*). Substitution of KCl for NaCl in the reaction buffer did not significantly alter the extent to which Ydj1p stimulated Ssb1/2p ATPase activity (not shown). Increasing the NaCl concentration from 20 to 50 mM in reactions abolished the effect of YDJ1p on Ssb1/2 ATPase activity, while 10-fold stimulation of Ssa1p and Ssa2p ATPase activity by Ydj1p was still observed (not shown). When the respective Hsp70 proteins were incubated with equimolar concentrations of Ydj1p and ATP was titrated into reaction mixtures, stimulation of Ssb1/2p ATPase activity was just above the level of background in our assays (Fig. 4*B*). Ydj1p, however, strongly stimulated the V_{max} of ATP hydrolysis catalyzed by both Ssa1p and Ssa2p at all ATP concentrations tested (Fig. 4*B*). Stimulation of Ssb1/2p ATPase activity by Ydj1p is very weak, and it is tempting to conclude that the two proteins do not interact. This may be premature, however, since DnaJ alone stimulates DnaK ATPase about 2-fold, but in combination with GrpE can synergistically stimulate ATPase activity up to 50-fold (Liberek *et al.*, 1991). No cytosolic eukaryotic GrpE homolog has been identified at this time. Thus, we conclude that the ATPase activity of both Ssa1p and Ssa2p is strongly regulated by YDJ1p, whereas Ydj1p alone is insufficient to significantly stimulate the ATPase of activity Ssb1/2p.

To extend these comparisons, the influence of Ydj1p on the ability of Ssa2p and Ssb1/2p to form a complex with F1β(1–51) was examined (Fig. 5). Ssa2p and Ssb1/2p both formed a complex with F1β(1–51) (see the legend to Fig. 5). Ssa2p, but not Ssb1/2p, could also form a complex with CMLA (not shown), confirming that Ssb1/2p has different substrate specificity than Ssa1p and Ssa2p (Gao *et al.*, 1991). Similar to results with Ssa1p, F1β(1–51)·Ssa2p complex formation was reduced only 18% by ATP (Fig. 5), whereas F1β(1–51)·Ssb1/2p complex formation was reduced 50% by ATP (Fig. 5). Addition of Ydj1p in combination with ATP, decreased F1β(1–51)·Ssa2p complex formation an additional 70% over ATP alone (Fig. 5). However, the combination of Ydj1p and ATP only reduced F1β(1–51)·Ssb1/2p complex formation by an additional 20% over ATP alone (Fig. 5). These data correlate with the effects of Ydj1p on the ATPase activity of Ssa2p and Ssb1/2p. Thus, the influence of Ydj1p on the ATPase and polypeptide binding activity of Ssa2p is almost identical to the influence on Ssa1p. However, there are significant differences in the effect of YDJ1p on the ATPase and polypeptide binding activities of Ssb1/2p compared to both Ssa1p and Ssa2p. Collectively, these data suggest that ATP and Ydj1p can have different effects on the chaperone-related activities attributed to members of the respective Hsp70 protein families present in the cytosol.

Polypeptide Binding to Ssa1p Reduces the Magnitude by Which Ydj1p Stimulates Its ATPase Activity—The *in vivo* chap-

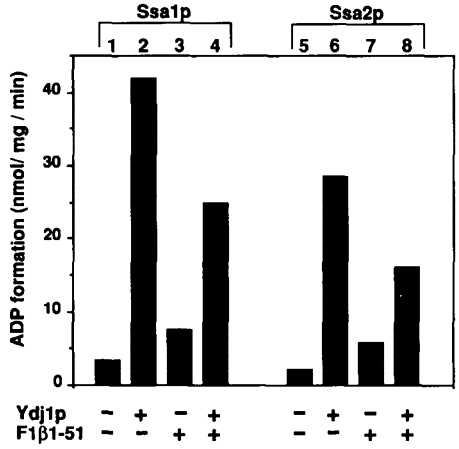

FIG. 6. **Reduced stimulation of Hsp70 ATPase activity by Ydj1p in the presence of F1β(1–51).** Respective Hsp70 homologs (0.45 μM) and [α-^{32}P]ATP (50 μM, 1.23 × 10^4 cpm/pM) were incubated in 20 μl of ATPase buffer for 5 min at 30 °C. Where indicated, Ydj1p (0.4 μM) and F1β(1–51) (100 μM) were present in reaction mixtures. For details pertaining to the composition of ATPase buffer and assay of ADP formation see "Materials and Methods."

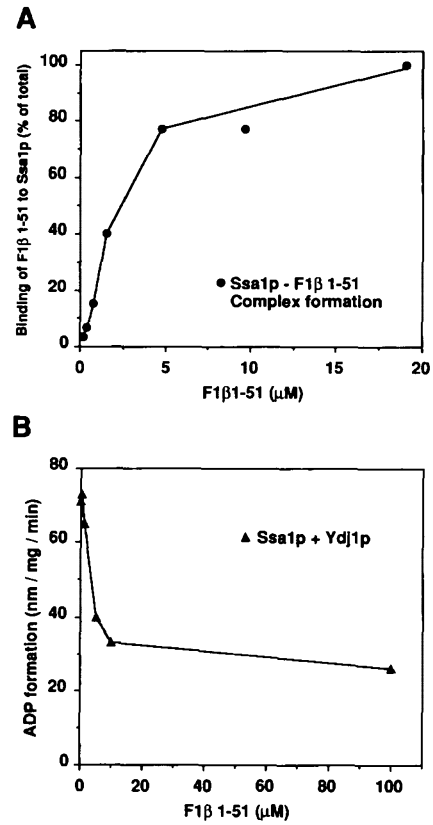

FIG. 7. **Saturation of F1β(1–51)-Ssa1p complex formation and maximal reduction of Ydj1p-dependent stimulation of Ssa1p ATPase activity occur at similar peptide concentrations.** *A*, titration of F1β(1–51) binding to Ssa1p. Ssa1p (0.45 μM) was incubated in 20 μl of gel shift buffer with ^{125}I-F1β(1–51) (as indicated, 9.6 × 10^3 cpm/pM) for 20 min at 30 °C. Complex formation was determined by native gel electrophoresis as described in the legend to Fig. 1. Shown is the quantitation of complex formation in percentage of total binding observed at 20 μM F1β(1–51), which is approximately 15% of total F1β(1–51) added to reaction mixtures. *B*, inhibition of Ydj1p-dependent stimulation of Ssa1p ATPase activity is saturable. Ssa1p (0.45 μM), Ydj1p (0.40 μM), ATP (50 μM, 1.23 × 10^4 cpm/pM), and F1β(1–51) (as indicated) were incubated in 20 μl of ATPase buffer at 30 °C for 5 min. ADP formation was determined as described in the legend to Fig. 5.

erone activity of Hsp70 requires ATP hydrolysis to release bound polypeptides. In the above experiments, we have examined the influence of Ydj1p on Hsp70 ATPase activity when the polypeptide binding site was presumably empty (Fig. 4). Therefore, we next tested the influence of Ydj1p on the ATPase activity of Ssa1p and Ssa2p under conditions where polypeptide substrate was bound (Fig. 6). Consistent with earlier reports on peptide-stimulated ATPase activity of Hsp70 proteins (Flynn *et al.*, 1989; Palleros *et al.*, 1991; Sedis and Hightower, 1992), addition of F1β(1–51) to the reaction mixture stimulated the ATPase activity of Ssa1p and Ssa2p about 2-fold (Fig. 6, *lane 1 versus lane 4*, and *lane 5 versus lane 7*). As noted previously, however (Sedis and Hightower, 1992), peptide-stimulated ATPase activity was very sensitive to salt conditions in ATPase reaction buffers (not shown). Surprisingly, when Ydj1p and F1β(1–51) were added in combination, there was a 40–45% decrease in the degree to which Ydj1p stimulated Ssa1p and Ssa2p ATPase activity (Fig. 6, *lane 1 versus lane 2* compared to *lane 1 versus lane 4*, and *lane 5 versus lane 6* compared to *lane 5 versus lane 8*). Similar results were obtained when peptide F1β(1–32) was employed as a substrate (not shown).

To determine if binding of F1β(1–51) by Ssa1p was related to reduced ability of Ydj1p to stimulate Ssa1p ATPase activity observed in the presence of F1β(1–51), saturation curves for these events were determined (Fig. 7). Saturation of F1β(1–51)·Ssa1p complex formation occurred at approximately 10 μM peptide (Fig. 7*A*). Reductions in the ability of Ydj1p to stimulate the ATPase activity of Ssa1p were also maximal at 10 μM F1β(1–51) (Fig. 7*B*). Thus, there is a strong correlation between binding of F1β(1–51) to Ssa1p and the reduced ability of Ydj1p to stimulate the ATPase activity of Ssa1p.

The domain on Hsp70 where DnaJ homologs interact has not been determined. If the Ydj1p binding domain is near the polypeptide binding domain, then substrate binding to Ssa1p could competitively block Ydj1p-Ssa1p interactions and thereby reduce stimulation of ATP hydrolysis. Therefore, the possibility that F1β(1–51) reduces the ability of Ydj1p to stimulate ATP hydrolysis by competitively interfering with Ydj1p-Ssa1p interactions needed to be excluded. This was accomplished by titrating Ydj1p into reaction mixtures containing Ssa1p and F1β(1–51), at concentrations where half-maximal complex formation and half-maximal reduction of Ydj1p-dependent ATP hydrolysis occurred (Fig. 8). The shape of the Ydj1p activation curve

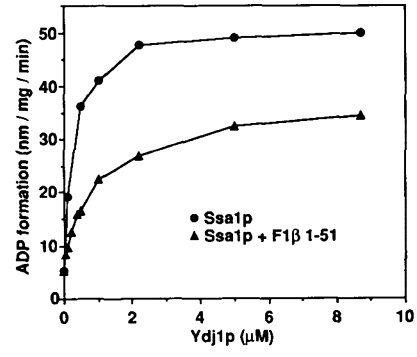

FIG. 8. **In the presence of F1β(1–51), titration of excess Ydj1p into Ssa1p ATPase reactions does not restore full stimulation of ATPase activity.** Ssa1p (0.45 μM), ATP (50 μM, 1.23 × 10^4 cpm/pM), Ydj1p (as indicated), and F1β(1–51) (2.5 μM) were incubated in 20 μl of ATPase buffer at 30 °C for 5 min. ADP formation was determined as described in the legend to Fig. 5.

was virtually unchanged in the presence of F1β(1–51,) except that the V_{max} of the reaction was reduced (Fig. 8). Increasing the Ydj1p:F1β(1–51) molar ratio did not reduce the extent to which F1β(1–51) reduced stimulation of Ssa1p ATPase activity by Ydj1p (Fig. 8). Consistent with these data, YDJ1p, at high

molar excess to both Ssa1p and polypeptide, does not block substrate binding to Ssa1p.[2] Thus, F1β(1–51) does not appear to competitively block interactions between Ydj1p and Ssa1p and thereby reduce stimulation of Ssa1p ATPase activity.

Instead, data in Figs. 6–8 support the interpretation that binding of F1β(1–51) to the polypeptide binding groove reduces the extent to which Ssa1p ATPase activity can be stimulated by YDJ1p.

DISCUSSION

Ssa1p, Ssa2p, and the Ssb Hsp70 proteins are present in the yeast cytosol at high concentrations under normal growth conditions and play specific roles in the maintenance of cellular metabolism (Deshaies *et al.*, 1988; Chirico *et al.*, 1988; Nelson *et al.*, 1992; Craig *et al.*, 1993). To uncover mechanisms that dictate the specificity of Hsp70 function, we have analyzed the influence of ATP and Ydj1p on the chaperone-related activities of purified Ssa1p, Ssa2p, and Ssb1/2p. The Ssa and Ssb Hsp70 proteins exhibited significant differences in response of their chaperone-related activities to Ydj1p. Additionally, polypeptide complexes that formed with Ssa1p and Ssa2p were less sensitive to ATP than similar complexes with several other Hsp70 proteins. These observations provide the first direct evidence that different Hsp70 homologs present in the same subcellular compartment exhibit differential sensitivity to a DnaJ homolog.

ATP had a range of effects on complex formation between different Hsp70 homologs and F1β(1–51). In the absence of ATP, the different Hsp70 homologs tested formed complexes with similar amounts of F1β(1–51). However, when ATP was present, complexes between F1β(1–51) and both Ssa1p and Ssa2p were significantly more stable than complexes with Ssb1/2p, BiP, and DnaK. It was recently reported that ATP alone is insufficient to dissociate complexes between Ssa1p or Ssa2p and prepro-α factor that are immunoprecipitated from translation lysates (Chirico, 1992). Consistent with our observations, dissociation of the Ssa1p/Ssa2p·prepro-α factor complex was dependent on both ATP and protein factors, possibly Ydj1p, in yeast cytosol (Chirico, 1992). All the above observations suggest that Ssa Hsp70 proteins have the ability to form complexes with polypeptides that have a long half-life in the cell. This would make Ssa1p and Ssa2p well suited for there role in intracellular protein transport pathways (Deshaies *et al.*, 1988; Chirico *et al.*, 1988) since the formation of long lived Hsp70·precursor protein complexes would prevent the premature release and misfolding of precursor proteins prior to entrance into protein translocation pathways. YDJ1p and possibly other factors, such as a GrpE-like protein, could assist Ssa1p and Ssa2p in this process by regulating the release of precursors at translocation sites.

Since Hsp70 proteins recognize and bind a broad range of polypeptide substrates (Flynn *et al.*, 1991), it is difficult to understand what dictates the specificity of Hsp70 function. One suggestion, based on work with *E. coli* DnaJ, is that DnaJ homologs increase the specificity of polypeptide binding by forming complexes with polypeptides and targeting them to DnaK (Wickner *et al.*, 1991). Even greater specificity of function could be elicited if Hsp70 molecules only interacted with specific DnaJ homologs. The field is still waiting for evidence that eukaryotic DnaJ homologs can bind to polypeptides. However, there is indirect evidence to suggest that specific DnaJ homologs interact with specific Hsp70 homologs (Brodsky *et al.*, 1993). The observation that Ssa1p and Ssa2p activity are strongly regulated by YDJ1p, whereas the activity of Ssb1/2p shows only a very weak response, provides direct evidence that Hsp70·DnaJ homolog interactions can have specific results.

The sharp differences in the response of the Ssa and Ssb Hsp70 proteins to Ydj1p raise the question of why these proteins, which are about 60% identical (Craig *et al.*, 1993), behave differently. One explanation is that the Ssb Hsp70 proteins do not interact with Ydj1p with the same affinity as the Ssa Hsp70 proteins. While another possibility is that both the Ssa and Ssb Hsp70 proteins interact with Ydj1p, but kinetic features of the Ssb1/2p ATP hydrolytic cycle limit the response to Ydj1p. Both possibilities are currently under investigation.

Several studies indicate that regulation of chaperone activity involves intramolecular communication between separate domains of Hsp70, which hydrolyze ATP and bind polypeptides (Flynn *et al.*, 1989; Liberek *et al.*, 1991; Sedis and Hightower, 1993). Data presented here indicate that events at the Ydj1p binding site are coupled to events at the ATPase and polypeptide binding domains of Ssa1p. This interpretation is based on the observation that titration of F1β(1–51) into reaction mixtures results in a dose-dependent decrease in the ability of Ydj1p to stimulate Ssa1p ATPase activity. This decrease in Ydj1p-dependent ATPase activity appears to result from direct binding of F1β(1–51) to Ssa1p and not competition between Ydj1p and F1β(1–51) for Ssa1p. To explain these results, we propose that in the absence of bound polypeptide the ATPase domain is uncoupled from the polypeptide binding domain and Ydj1p binding alters the conformation of Ssa1p to maximally stimulate ATPase activity. However, when F1β(1–51) occupies the polypeptide binding groove, the flexibility of Ssa1p is reduced and this limits the ability of Ydj1p to fully stimulate ATPase activity. Alternatively, the formation of a F1β(1–51)·Ydj1p·Ssa1p ternary complex (Langer *et al.*, 1992) may also reduce stimulation of Ssa1p ATPase activity by increasing the half-life of F1β(1–51)·Ssa1p complexes and thereby slowing the cycle of ATP hydrolysis and substrate release. Future studies designed to elucidate the mechanism for stimulation of Hsp70 ATPase activity by DnaJ homologs will provide a test for these possibilities.

Ydj1p can stimulate the ATP-dependent release of model polypeptides bound to Ssa1p and Ssa2p. In contrast, DnaJ can stabilize the binding of polypeptides to DnaK (Langer *et al.*, 1992). When comparing these results it should be noted that different substrate proteins were used in these studies. Experiments with Ydj1p employed the permanently unfolded protein CMLA and F1β(1–51), a presequence peptide that is not predicted to exhibit secondary or tertiary structure in aqueous solution (Hoyt *et al.*, 1991). Studies with DnaJ employed both CMLA and folding intermediates of rhodanese (Langer *et al.*, 1992). DnaJ can form a high affinity complex with rhodanese but not with CMLA (Langer *et al.*, 1992). Similarly, Ydj1p has a low affinity for linear polypeptides (Cyr and Douglas, 1992). Stabilization of substrate binding appears to result from simultaneous binding of DnaJ to rhodanese and DnaK to form a ternary complex (Langer *et al.*, 1992). This does not occur to a significant extent if CMLA is substituted for rhodanese (Langer *et al.*, 1992). The ability of DnaJ to stabilize complexes between polypeptides and DnaK is thus dependent on the conformation of the substrate protein. We are currently testing the possibility that Ydj1p stabilizes complex formation between the Ssa and Ssb Hsp70 proteins and protein folding intermediates.

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