Hop Modulates hsp70/hsp90 Interactions in Protein Folding

(Received for publication, October 14, 1997, and in revised form, November 7, 1997)

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Hop is a 60-kDa protein characterized by its ability to bind the two chaperones, hsp70 and hsp90. We have tested the function of Hop using an assay for the refolding of denatured firefly luciferase. We show that Hop is involved in the process of refolding thermally denatured firefly luciferase in rabbit reticulocyte lysate. Hop also stimulates refolding by hsp70 and Ydj-1 in a purified refolding system. Hsp90 can also stimulate refolding, and optimal refolding is observed in the presence of both Hop and hsp90. Similar stimulation was observed when Hop was replaced by its yeast homolog Sti1. In assays of the binding of Hop to hsp70 and hsp90, Hop preferentially forms a complex with ADP-bound hsp70, and this process is unaffected by the presence of hsp90. Hop does not alter the ATPase activity or the rate of ADP dissociation of hsp70. Hop also appears to bind to the ADP-bound form of hsp90, blocking the ATP-dependent conversion of hsp90 to a form capable of interacting with p23. Conversely, once p23 is bound to hsp90, Hop binding is diminished. These results confirm that Hop provides a physical link between hsp70 and hsp90 and also indicate that Hop modulates the activities of both of these chaperone proteins.

The molecular chaperones hsp70 and hsp90 are two of the most prominent heat shock proteins in the eukaryotic cytosol. Of the two, hsp70 is the more widely studied and has been extensively characterized in bacteria (DnaK), in yeast (Ssa and Ssb), and in several compartments of higher eukaryotes. hsp70s are composed of two domains as follows: a 45-kDa, amino-terminal nucleotide-binding domain and a 25-kDa, carboxyl-terminal peptide-binding domain. Through a cycle of ATP binding, hydrolysis, and nucleotide exchange, denatured proteins are alternately bound and released to effect protein folding (1, 2). Substrates bind transiently to the ATP-bound form of hsp70, but when ATP is hydrolyzed the binding is stabilized (3). Release of the substrate occurs when ADP is exchanged for ATP (4). hsp70 function is modulated by members of the hsp40 family in higher eukaryotes, Ydj1 in yeast, and DnaJ in bacteria. These proteins are known to stimulate the ATPase activity of their respective hsp70s (5, 6) and are required for hsp70-mediated refolding of denatured substrate proteins (7, 8). In addition, some studies suggest that hsp40s may act independently to recognize and bind unfolded polypeptides to prevent aggregation and target them to hsp70 (9–11). In bacteria, DnaK function is also dependent on a nucleotide exchange factor, GrpE, but no eukaryotic cytoplasmic homolog has been identified (6, 12).

hsp90 has also been shown to play a role in protein folding and in the functional maturation of a number of kinases and receptors. hsp90 is often studied as a chaperone necessary for the maturation of progesterone and glucocorticoid receptors, and in these systems an intermediate and a mature state during complex assembly have been described (13, 14). The intermediate complex contains hsp70 and Hop (also called p60), the human homolog of the yeast stress-induced protein STI1, plus some hsp90 and Hip (also called p48) (15, 16). This progresses to a complex containing less hsp70 and Hop, more hsp90, and the hsp90-binding proteins p23 and one of three large immunophilins (FKBP51, FKBP52, or cyclophilin 40). The appearance of this complex correlates with the final step of receptor maturation (17–19). The mechanistic details of hsp90 function are still unclear. However, it does appear that similar hsp90 complexes are utilized in the maturation of a number of different target proteins (20, 21). The functions of the accessory proteins that bind to hsp90 are only beginning to be understood.

Hop is an abundant, stress-induced protein (22) associated with hsp90 and hsp70 in dynamic complexes in reticulocyte lysate and found as a component of intermediate steroid receptor complexes (15, 16). Hop has been shown to be necessary for the in vitro assembly of steroid receptors with hsp90 (15, 23). Its yeast homolog, STI1 (24), is almost entirely complexed with hsp90 (25). Therefore, it seems likely that this protein functions along with hsp90. Since hsp70 and hsp90 are not associated with one another in its absence, yet each associates individually or in a ternary complex with Hop, it appears that Hop is responsible for the organization of this complex (15, 17, 26). Hop has recently been reported to stimulate the ATPase activity of hsp70 and to increase hsp70’s affinity for ATP, thus serving the role of nucleotide exchange factor for hsp70, analogous to the role of GrpE in prokaryotic systems (27). In assays of passive chaperoning activity, Hop does not show any productive interactions with the denatured substrates citrate synthase or ß-galactosidase (28, 29). However, one recent study suggests that Hop is associated with luciferase during the refolding process in reticulocyte lysate (30).

An assay for the chaperone-mediated refolding of thermally denatured firefly luciferase has been described previously. In this system, the chaperones hsp70 and Ydj-1 are absolute requirements for the refolding process, and hsp90 can enhance refolding under some conditions (8). We used this assay to investigate the role of Hop in protein refolding. Hop is involved
in refolding in rabbit reticulocyte lysate and can significantly stimulate refolding using purified chaperones. Hop binds to hsp70 and hsp90 in a nucleotide-dependent manner and may also modulate the functions of these two chaperones.

### EXPERIMENTAL PROCEDURES

#### Antibody Preparation

Monoclonal antibody F5 was prepared against avian p60/Hop as described previously (16). F5 antibody cross-reacts with human Hop. Mouse monoclonal antibody 4F3 was prepared against chicken hsp90 and does not cross-react with rabbit hsp90 (31). Mouse monoclonal antibody J33 was prepared against human p23 as described previously (32). Mouse monoclonal antibody BB70 was prepared against avian hsp70 complexed with hsp90 as described previously (16). BB70 cross-reacts with both free and complexed human hsp70. A rabbit antiserum against Hdj-1 was supplied by W. J. Walsh and has been described previously. This antisera cross-reacts with several J proteins important to the refolding process. Antibody ST2 is a mouse monoclonal IgG2 prepared against ST11. Puriﬁed ST11 (see below) was used as the antigen and for screening by enzyme-linked immunosorbent assay and Western blotting. Balb/c mice were injected subcutaneously with 100 μg of antigen in Freund’s incomplete adjuvant. Splenocytes were fused with myeloma cell line Sp2/0 Ag 14, and hybridomas were selected and screened by conventional methods.

#### Purification of hsp90—hsp90 was prepared by the overexpression of human hsp90α in SF9 cells using the system of Alnemri and Litwack (33). The preparation was as described previously (34). Cell lysates were fractionated by DEAE-cellulose column chromatography, followed by heparin-agarose column chromatography, and then Mono Q FPLC. The preparation was greater than 99% pure as assessed by densitometry of SDS-PAGE gels. Western blot analysis using rabbit antisera against Hdj-1 showed no contamination by J proteins. Protein concentration was determined by amino acid analysis.

#### Purification of hsp70—hsp70 was prepared by the overexpression of human hsp70 in SF9 cells using the system of Alnemri and Litwack (33). The purification was as described previously for avian hsp70 (8). Cell lysates were fractionated by DEAE-cellulose column chromatography followed by ATP-agarose column chromatography. This was precipitated using ammonium sulfate (75% saturation), and the redissolved fraction was loaded on a DEAE-cellulose column that was washed 4 times with TB, extracted into SDS sample buffer, and probed using hsp90 antisera. The preparation was greater than 98% pure as assessed by densitometry of SDS-PAGE gels.

#### Purification of Hop—Human Hop expressed in bacteria was prepared essentially as described previously (35). Bacterial lysates were fractionated by DEAE-cellulose chromatography followed by hydroxylapatite column chromatography. Additional purification was achieved by fractionating the pool from hydroxylapatite on a Mono Q FPLC column. Cell lysates were fractionated with a linear gradient of 0–0.5 M KCl. The fractions containing Hop were pooled, dialyzed into 10 mM Tris-HCl, 1 mM DTT, and 1 mM EDTA, pH 7.5, and stored at −70 °C. The preparation was approximately 97% pure as assessed by densitometry of SDS-PAGE gels. Western blot analysis using rabbit antiserum against Hdj-1 showed no contamination by J proteins. Protein concentration was determined by amino acid analysis.

#### Purification of Ydj1—A bacterial expression system for Ydj1p was generously supplied by Dr. Avrom Caplan and has been described previously (36). Bacterial lysates were fractionated by DEAE-cellulose column chromatography followed by hydroxylapatite column chromatography. The preparation was approximately 90% pure as assessed by densitometry of SDS-PAGE gels. Protein concentration was determined by amino acid analysis.

#### Purification of ST11—The DNA for ST11 of Saccharomyces cerevisiae was obtained from Elizabeth Craig (24). This was placed in a pET-11 vector and expressed in Escherichia coli. Cell lysates were prepared by sonication in 3 volumes of 10 mM Tris-HCl, 1 mM EDTA, 10 mM monothioglycerol, pH 7.5, and the proteasome inhibitors pepstatin, 2 μg/ml leupeptin, 2 μg/ml, and 4-2-aminoethylbenzenesulfonyl ﬂuoride, 1 mM EDTA. ST11 was extracted as a soluble protein, and after centrifugation, it was loaded on a DEAE-cellulose column that was washed with 10 mM Tris-HCl, 1 mM EDTA, 10 mM thioglycerol, pH 7.5, and eluted with a 0–0.4 M KCl gradient. Fractions containing ST11 were pooled and loaded on a hydroxylapatite column that was washed with 10 mM potassium phosphate, 10 mM thioglycerol, and 0.1 M KCl, pH 7.4, and eluted with a gradient of 10–400 mM potassium phosphate. Fractions containing ST11 were pooled and loaded onto a 16/60 Superdex 200 sizing column and eluted with 10 mM Tris-HCl, 1 mM DTT, and 0.25 μM KCl, pH 7.5. The fractions containing ST11 were pooled and dialyzed into 10 mM Tris-HCl, 1 mM DTT and 1 mM EDTA, pH 7.5, and stored at −70 °C. The preparation was greater than 98% pure as assessed by densitometry of SDS-PAGE gels. Protein concentration was determined by amino acid analysis.

#### Purification of p23—The bacterial expression and puriﬁcation of human p23 has been described (18). The soluble fraction of bacterial lysate was fractionated by DEAE-cellulose column chromatography followed by phenyl-Sepharose (hp1660) FPLC, dialyzed into 10 mM Tris-HCl, 1 mM DTT, and 1 mM EDTA, pH 7.5, and stored at −70 °C. The preparation was greater than 95% pure as assessed by densitometry of SDS-PAGE gels.

#### Buffers—Tris buffer (TB) was as follows: 10 mM Tris-HCl, pH 7.5, 3 mM MgCl2, 50 mM KCl, and 2 mM DTT. Stability buffer (SB) was as follows: 25 mM Tricine-HCl, pH 7.8, 8 mM MgSO4, 0.1 mM EDTA, 10 mg/ml bovine serum albumin, 10% glycerol, and 0.25% Triton X-100.

#### Luciferase Refolding Assay—Luciferase refolding assays were performed as described previously (8). Firefly luciferase, 100 nM in SB, was heat-denatured at 40 °C to −0.2% of its original activity. This was diluted 10-fold into reticulocyte lysate or a refolding mixture containing purified chaperone proteins, 2 μM ATP, and an ATP-regenerating system. The refolding mixture was incubated at 25 °C to promote refolding, and at the indicated times following addition of denatured luciferase, aliquots were removed, and luciferase activity was measured in a luminometer. The luciferase activities were expressed as a percent of control samples of the same luciferase concentration that was not denatured.

#### ATPase Assay—The hsp70-catalyzed hydrolysis of ATP was measured essentially as described previously (38). Assays containing 2 μM hsp70, 20 μM ATP, and 5 μCi of [γ-32P]ATP in 50 μl were incubated at 37 °C for 30 min. A 10-μl aliquot was removed before the start of each incubation, and additional aliquots were removed after 10, 20, and 30 min of incubation and added to 500 μl of an acidified charcoal suspension. The charcoal, along with the nucleotides, was pelleted, and the amount of free phosphate in the supernatant was measured by liquid scintillation counting.

#### Hop/ST11 Binding to hsp90—Hop and ST11 were measured by combining the appropriate proteins (−10 μg each) in 200 μl of buffer containing 10 mM Tris-HCl, 5 mM MgCl2, 50 mM KCl, and 1 mM DTT, pH 7.5. In some cases, ATP or ADP was included (see figure legends). The samples were incubated for 30 min at 30 °C, chilled on ice, and combined with 25-μl pellets of protein A-Sepharose containing either antibody F5 against Hop or ST2 against ST11. After incubation for 1 h in ice, the resin pellets were washed, extracted into SDS sample buffer, and proteins were resolved by SDS-PAGE as described previously (37).

#### Nucleotide Exchange Assay—Samples containing 1 μM hsp70, −0.2 μCi of [γ-32P]ATP, and 1 μM total ATP in 25 μl of TB were incubated with 250 nM Hop at 37 °C for 30 min to allow hsp70 to bind and hydrolyze the ATP. These were cooled on ice and then dialyzed 2-fold into TB containing some combination of 1 mM free ATP and/or 0.1 mg/ml bovine carboxymethylated-a-lactalbumin (CMLA) and incubated again at 37 °C to allow release of the labeled nucleotide from hsp70. A 9-μl aliquot was removed prior to the start of each incubation, and additional aliquots were removed after 1, 2, 4, 8, and 6 min of incubation and filtered through nitrocellulose filters. Each filter was washed twice with 1 ml of TB, and the amount of bound nucleotide on each filter was determined by liquid scintillation counting.

#### Hop and p23 Binding to hsp90—The abilities of Hop and p23 to bind to hsp90 were measured by combining 220 nM hsp90 with 10 μM ATP or 10 μM ADP in 200 μl of 20 mM Na3MoO4, 0.01% Nonidet P-40, 5 mM MgCl2, and 10 mM Tris-HCl, pH 7.5. Samples were incubated for 30 min at 30 °C and then chilled and incubated an additional 10 min on ice. Hop and/or p23 were added to the samples either before or after the 30 °C incubation. Samples were combined with 25-μl pellets of protein A-Sepharose conjugated with either antibody J33 against p23 or F5 against Hop and incubated for 90 min on ice. The resin pellets were washed 4 times with TB, extracted into SDS sample buffer, and proteins were resolved by SDS-PAGE as described previously (37).

### RESULTS

#### Hop Is Involved in Reticulocyte Lysate-mediated Luciferase Refolding—To determine whether Hop is involved in the process of refolding firefly luciferase in rabbit reticulocyte lysate,
we added thermally denatured luciferase to a refolding mixture containing reticulocyte lysate dialyzed in TB, with ATP, and an ATP-regenerating system and incubated the reaction at 25 °C to promote refolding. We inhibited Hop's function in reticulocyte lysate using monoclonal antibody F5 (15), then used this lysate in refolding experiments (not shown). As increasing amounts of F5 antibody are added to the lysate used to refold denatured firefly luciferase, the initial rate of the refolding reaction (measured after 15 min) is progressively reduced by as much as 25%. As the time course of refolding proceeds, the inhibition caused by F5 antibody gradually decreases until there is about a 10% difference in the extent of refolding after 60 min.

We also used lysates treated with high salt (0.4 M KCl) followed by immune depletion of Hop for luciferase refolding (Fig. 1, A and B). High salt treatment of reticulocyte lysate followed by buffer exchange decreases the initial rate of refolding by about 40%. Immune depletion of Hop from high salt-treated lysate further reduces the initial rate of luciferase refolding compared with control preparations by up to 25% (Fig. 1, A and B). After two rounds of depletion, Hop was undetectable by Western blot analysis in the reticulocyte lysate preparations. Supplementation Hop-depleted lysate with purified human Hop at 0.1, 0.3, or 1 μM restores the refolding ability of the lysate (Fig. 1A), but addition of purified Hop to control lysate that has not been depleted of Hop causes a small loss in refolding ability (Fig. 1B), most likely the result of exceeding the optimal concentration of Hop. After 80 min of refolding time, the extent of refolding in control preparations is not significantly different than in untreated lysate. However, considerably less luciferase is refolded during 80 min in Hop-depleted preparations (Fig. 1B). When Hop is depleted from reticulocyte lysate at normal salt concentrations, the rate of refolding is reduced by about ½ (not shown). In this case, the addition of purified Hop only partially restores the lost activity. The most probable explanation for this result is that other chaperones such as hsp70 and hsp90 which are known to associate with Hop and play a role in refolding are being coprecipitated and are no longer available for refolding. These results show that the refolding of luciferase in reticulocyte lysate is influenced by Hop, but Hop is clearly not an essential component of the refolding machinery.

Purified Chaperones Demonstrate That Hop Participates with hsp70, hsp90, and Ydj in Refolding—We used purified chaperone proteins in the luciferase refolding assay to characterize further Hop's role in refolding. Thermally denatured luciferase was added to a refolding mixture containing purified chaperone proteins, ATP, and an ATP-regenerating system and allowed to refold at 25 °C; the results are shown in Fig. 2A. In the absence of either hsp70 or Ydj, no individual chaperone or combination of chaperones is able to mediate luciferase refolding. When hsp70 and Ydj are both present in the refolding reaction, a significant amount of luciferase refolding occurs, as described previously (8), and this can be further stimulated by the addition of either Hop or hsp90. We see a dramatic stimulation in the rate and extent of luciferase refolding when both Hop and hsp90 are added to hsp70 and Ydj. This effect is most pronounced during the early stages of refolding (30 min) where greater than 10-fold stimulation is observed compared with a 2–3-fold stimulation for Hop or hsp90 alone. Identical results are obtained when STI1 is substituted for Hop in this assay (Fig. 2B).

The optimal concentration of Hop for a particular refolding reaction is in large part determined by hsp90. In the absence of hsp90, the optimal concentration of Hop in refolding reactions is greater than 100 nM (Fig. 3A). When hsp90 is present, additional stimulation of refolding occurs along with a decrease in the optimal concentration for Hop to a range of 10–100 nM. This 10-fold decrease in the most effective concentration makes Hop optimum for refolding at a concentration similar to that of the substrate luciferase (10 nM). STI1 showed a very similar pattern of concentration dependence with a higher concentration (1 μM) being most effective in the absence of hsp90 and low to moderate concentrations (10–100 nM) being more effective when hsp90 is present (not shown).

Hop and hsp90 also have effects on the concentration of
hsp70 required for the refolding process as shown in Fig. 3B. The optimal concentration range for hsp70 is quite narrow since excess hsp70 is very inhibitory. hsp70 is most effective at a concentration of approximately 350 nM for refolding in the presence of 80 nM Ydj. Addition of either Hop or hsp90 causes a slight increase in the requirement for hsp70 in addition to providing a stimulation to the refolding process at most hsp70 concentrations. When Hop and hsp90 are added together to the refolding mixture, the effective concentration range for hsp70 is shifted to a higher level and dramatically broadened, yielding a highly effective refolding mixture at hsp70 concentrations from 350 nM up to 4 μM.

**Hop Does Not Affect hsp70's ATPase Activity or Nucleotide Exchange Rate**—We were interested in determining Hop’s mechanism of action in the luciferase refolding assay, particularly those assays in which hsp90 was absent. Gross and Hessefort (27) have recently isolated and characterized a Hop homolog from rabbit reticulocyte lysate which they call RF-hsp70. They found that this protein stimulated the ATPase activity and nucleotide dissociation rate of hsp70. We wanted to confirm these activities using highly purified human Hop. Fig. 4 shows the results of an ATPase assay in which the hydrolysis of ATP by hsp70 is determined by measuring free phosphate release over a time course in the presence or absence of Hop. Hop, which is known to stimulate the ATPase activity of hsp70 (5, 6), was used as the positive control. ATP hydrolysis is clearly enhanced in the presence of either 25 or 250 nM Ydj, but the presence of 0.22 or 2.2 μM Hop did not alter hsp70’s ATPase activity either in the absence or presence of Ydj. In the absence of hsp70, no significant hydrolysis of ATP was catalyzed by Ydj or Hop.

Consistent with this result, we have found that Hop binds
preferrably to the ADP-bound form of hsp70 (see below). A factor that stimulates ATP hydrolysis would be expected to associate with the ATP-bound form of the protein to then effect hydrolysis. Several studies have shown that hsp70 and hsp90 associate with Hop to form a three-way complex both in cell extracts and using purified proteins (14, 15, 26). Fig. 5A shows the results of immune precipitations of Hop from a mixture containing purified Hop, hsp70, and hsp90 under several different nucleotide conditions. In lane 1, no exogenous nucleotide was added to the reaction and hsp70 binding to Hop was maximal. In lane 2, hsp70 binding was abolished by the addition of 10 μM ATP to the reaction. When 10 μM, 100 μM, or 1 mM ADP was added in combination with 10 μM ATP, hsp70 binding to Hop was progressively restored (lanes 3–5). Lanes 6 and 7 show the amount and purity of Hop and hsp70 added to the binding reactions.

Hop binding to hsp70 is unaffected by hsp90 as shown in Fig. 5B where binding is progressively lost as the ATP concentration is increased over a range of 0–100 μM in the presence or absence of hsp90. The binding of hsp90 to Hop does not appear to be affected by nucleotides under these conditions. However, when hsp90 is incubated under the proper circumstances to allow its ATP-induced conformation, Hop no longer binds to it (Fig. 7).

Hop binding to hsp70 in the presence of 10 μM ATP can be accomplished if Ydj is also added into the reaction mixture. In Fig. 5C, lanes 1 and 2 show the binding of Hop to hsp70 in the absence or presence of 10 μM ATP. Lanes 3 and 4 are identical except that 11 nM Ydj was added; this is approximately 1/100 the concentration of hsp70. In lanes 5 and 6, 110 nM Ydj was added. Lanes 7–10 show the amount and purity of Ydj, hsp90, hsp70, and Hop that were added to each sample. The binding of Hop to hsp70 which is lost upon the addition of 10 μM ATP is progressively restored with the addition of increasing amounts of Ydj. It seems probable that when ATP is bound to hsp70, Ydj stimulates hsp70 to hydrolyze the ATP, and Hop then binds to the ADP-bound hsp70. This is inconsistent with the previous hypothesis that Hop acts as a nucleotide exchange factor for hsp70 by increasing its ATPase activity, thus stimulating luciferase refolding.

STI1 behaves similarly to Hop in its ability to associate with hsp70. Fig. 5D shows the results of an immune precipitation of STI1 with antibody ST2 from a mixture of hsp70, hsp90, and STI1. When neither nucleotide or 1 mM ADP is added to the reaction mixture (lanes 1 and 6), binding of hsp70 to STI1 is maximal. As ATP is added in increasing amounts from 1 μM to 1 mM, binding of hsp70 to STI1 is progressively lost (lanes 2–5). This is identical to the behavior of hsp70 binding to Hop.

We also investigated Hop's role in promoting nucleotide dissociation from hsp70. Fig. 6 shows results from a nucleotide exchange assay in which hsp70 was preincubated with or without Hop in the presence of [α-32P]ATP and allowed to bind and hydrolyze the ATP. The mixture was then diluted into TB containing unlabeled ATP and/or the protein substrate CMLA to promote release of the labeled nucleotide. In the absence of added ATP, there is no significant release of labeled ADP from the hsp70. When ATP is added to the samples, bound ADP is released into solution, and this rate of release can be increased by adding a substrate for the hsp70 molecules, CMLA. The presence or absence of Hop has no effect on the rate of release of ADP from hsp70 under any of the conditions tested.

Hop and p23 Bind to and Stabilize Distinct Forms of hsp90—We were interested in learning more about the interaction of Hop with hsp90. hsp90 is often found associated with a small protein of unknown function, named p23, which is essential for the functional maturation of progesterone and glucocorticoid receptor complexes (18, 39). In order for hsp90 to bind to p23, it must first undergo an ATP-dependent conversion as described by Sullivan et al. (34). We wanted to determine whether Hop could also associate with this form of hsp90 and what effect, if any, the binding of one of these proteins had on the binding of the other.

Fig. 7 shows the results of an assay of Hop/p23 binding to hsp90 with different nucleotides present. p23 does not associate with nucleotide-free or ADP-bound hsp90 (lanes 1–2), but when hsp90 is bound to ATP at 30 °C, p23 is able to bind (lane 3). Note that the conditions include 20 mM sodium molybdate and 0.01% Nonidet P-40 which potentiate the conversion of hsp90 to the p23 binding state (34). If Hop is added during the 30 °C incubation, it prevents the binding of hsp90 to p23 (lane 4). When Hop and p23 are added simultaneously to hsp90 which has already been incubated at 30 °C with ATP, Hop is unable to prevent p23 from binding to hsp90 (lane 5). Thus Hop does not appear to directly block p23 binding. In contrast to p23, Hop binds to nucleotide-free or ADP-bound hsp90 (lanes 6–7), and binding to the ATP-bound form of hsp90 is reduced (lane 8). The binding of Hop to hsp90 in the presence of ATP can be reduced further by adding p23 during the 30 °C incubation to stabilize the ATP-bound state (lane 9). The addition of p23 and ATP does not interfere with the binding of Hop to hsp90 when the initial incubation is carried out on ice rather than at 30 °C (lane 10). This condition does not allow the ATP-dependent conversion of hsp90 (34). To rule out the possibility that some treatments may mask antibody epitopes and yield misleading results, additional experiments were conducted using both antibodies sequentially (not shown). In no case were both p23 and Hop bound to hsp90 in the same complex. These data show that Hop binds preferentially to the ADP-bound form of hsp90 and prevents its conversion to the p23 binding state, rather than simply blocking the binding site for p23. Although Sti1 from yeast is also able to bind to human hsp90, it does not prevent hsp90’s conversion to a form capable of binding to p23 (not shown).
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A

B

C

D

FIG. 5. Hop and Sti1 bind to hsp90 and the ADP-bound form of hsp70. A, the binding of hsp70 and hsp90 to Hop was tested under different nucleotide conditions. Samples containing 0.6 μM Hop, 1 μM hsp70, and 0.7 μM hsp90 were incubated in the presence of the following nucleotides: none (lane 1), 10 μM ATP (lane 2), 10 μM ADP (lane 3), 10 μM ATP and 100 μM ADP (lane 4, 10 μM ATP and 1 mM ADP (lane 5). Lane 6 shows the amount of Hop in each incubation, and lane 7 shows the amount of hsp70 in each incubation. B, the binding of hsp70 to Hop in the presence of increasing amounts of ATP was tested in the presence and absence of hsp90. Incubations contained 1 μM Hop, and 0.5 μM hsp70 in the presence of the following amounts of ATP: none (lanes 1 and 2), 0.1 μM ATP (lanes 3 and 4), 1 μM ATP (lanes 5 and 6), 10 μM ATP (lanes 7 and 8), 100 μM ATP (lanes 9 and 10). In addition, lanes 1, 3, 5, 7, and 9 contained 0.7 μM hsp90. C, the binding of hsp70 to Hop in the presence of Ydj was investigated. Incubations contained 0.8 μM Hop, 1.1 μM hsp70, and 0.55 μM hsp90 in the presence of the following additions: 0.1 mM ADP (lane 1), 2 mM ATP (lane 2), 0.1 mM ADP and 11 mM Ydj (lane 3), 2 mM ATP and 11 mM Ydj (lane 4), 0.1 mM ADP and 110 mM Ydj (lane 6). In addition, lanes 1, 3, 5, 7, and 9 contained 0.7 μM hsp90. D, the ability of hsp70 to bind to Sti1 was showed in the presence of hsp90 and nucleotides. Samples containing 0.7 μM Sti1, 1 μM hsp70, and 0.4 μM hsp90 were incubated in the presence of the following nucleotides: none (lane 1), 1 μM ATP (lane 2), 10 μM ATP (lane 3), 100 μM ATP (lane 4), 1 mM ATP (lane 5), 1 mM ADP (lane 6). Samples were incubated at 30 °C for 30 min, precipitated with antibodies to Hop/Sti1, run on 10% gels, and stained with Coomassie Blue.

Discussion

Hop is implicated as an important component of the protein folding process by its known association with, and ability to mediate a complex between hsp70 and hsp90, two of the most prominent molecular chaperones (15, 16). It participates with hsp70 and hsp90 as the minimal components needed to assemble glucocorticoid receptor complexes (40). In addition, Hop is found associated with denatured luciferase during the refolding process in reticulocyte lysate (30, 41). It is commonly thought that this ability to mediate a ternary complex between chaperones is the reason Hop is associated with denatured proteins, but the exact role of Hop in this process has remained unclear despite some recent clarifications of the mechanisms of both hsp70 and hsp90.

hsp90 and several of its accessory proteins support specific chaperoning activity (28, 29). They are able to suppress aggregation of denatured β-galactosidase or citrate synthase and maintain these proteins in a refoldable state. However, Hop is inactive in these assays suggesting that it does not perform a chaperone function directly (28, 29). The luciferase refolding assay used in the present experiments is more demanding than the assays cited above due to its energy-requiring refolding process and the presence of multiple chaperones which may allow many interesting and unexplored interactions of Hop. Under the heat denaturation conditions used here, luciferase is not aggregated, but its exact structure or extent of denaturation has not been defined. It is possible that unfolding as well as productive folding are both components of the refolding process of denatured luciferase.

Results from the luciferase refolding assay show that while Hop is not an essential component of the refolding machinery, it does have a role in refolding proteins in a complex environment such as reticulocyte lysate, where inhibition of Hop’s function with a monoclonal antibody or immune depletion of Hop results in a decline in refolding efficiency. In a defined environment, using purified chaperones to refold luciferase, Hop is able to significantly stimulate the rate of refolding by hsp70 and Ydj. A much more dramatic effect is seen when hsp90 is also present in the refolding mixture. It is not surprising that Hop can stimulate refolding when both hsp70 and hsp90 are present since it is presumed to act as a mediator of the two (15, 26). What was not expected is that Hop can stimulate refolding in the absence of hsp90, meaning that Hop can modulate hsp70/Ydj function in some manner independent from hsp90 or that Hop itself is able to chaperone some step in luciferase refolding.

Hop has not been shown to have any independent chaperoning activity, so we investigated several possible effects Hop could have on hsp70. In contrast to a recent report using a Hop homolog from rabbit reticulocyte lysate (27), we were unable to detect a Hop-induced stimulation of hsp70’s ATPase activity in the presence or absence of Ydj. Also in contrast to this previous report, we saw no effect of Hop on ADP dissociation from hsp70 or ATP binding by hsp70 (results not shown). Consistent with these results, we found that Hop binds only to ADP-bound hsp70 in immune precipitations of the purified proteins, meaning that Hop is not associated with hsp70 at the proper time to stimulate ATP hydrolysis. Binding to ATP-bound hsp70 only 7–10 show the purity of the Ydj, hsp90, hsp70, and Hop preparations used in these experiments. D, the ability of hsp70 to bind to Sti1 was showed in the presence of hsp90 and nucleotides. Samples containing 0.7 μM Sti1, 1 μM hsp70, and 0.4 μM hsp90 were incubated in the presence of the following nucleotides: none (lane 1), 1 μM ATP (lane 2), 10 μM ATP (lane 3), 100 μM ATP (lane 4), 1 mM ATP (lane 5), 1 mM ADP (lane 6). Samples were incubated at 30 °C for 30 min, precipitated with antibodies to Hop/Sti1, run on 10% gels, and stained with Coomassie Blue.
FIG. 6. Hop does not alter nucleotide exchange by hsp70. The rate of nucleotide exchange (ADP for ATP) by 1 μM hsp70 was determined in the presence of the following additions: none (×); 1 mM ATP (open squares); 0.1 mg/ml CMLA (open triangles); 1 mM ATP and 0.1 mg/ml CMLA (open circles); 0.25 μM Hop (+); 0.25 μM Hop and 1 mM ATP (filled squares); 0.25 μM Hop and 1 mg/ml CMLA (filled triangles); 0.25 μM Hop, 1 mM ATP, and 0.1 mg/ml CMLA (filled circles). The symbols for no addition (×), CMLA (open triangles), Hop (+), and Hop + CMLA (filled triangles) are mixed together and define the four horizontal lines showing no nucleotide exchange at the top of the figure. The labeled ADP which remained bound to hsp70 was isolated on nitrocellulose filters and quantitated by liquid scintillation counting.

FIG. 7. Hop and p23 bind to and stabilize distinct forms of hsp70. The binding of hsp70 (220 nM) to p23 (4.2 μM) and Hop was investigated in a two-step process with Hop and p23 incubated separately or together, under different nucleotide conditions. Lanes 1–5 measure the binding of hsp90 to p23. hsp90 was incubated at 30 °C for 30 min without nucleotide (lane 1), with 5 mM ADP (lane 2), 5 mM ATP (lane 3), ATP plus 2.6 μM Hop (lane 4), or with ATP at 30 °C and subsequent addition of Hop at 0 °C (lane 5). p23 was then added at 0 °C for 30 min, and complexes were recovered using antibody to p23. Lanes 6–10 measure the binding of hsp90 to Hop. hsp90 was incubated at 30 °C for 30 min without nucleotide (lane 6), with ADP (lane 7), with ATP (lane 8), or with ATP plus p23 (lane 9). The sample for lane 10 was treated identically to the sample in lane 9 but without the 30 °C incubation. 430 nM Hop was added at 0 °C after the 30 °C incubation of each sample, and complexes were recovered using antibody to Hop. Samples were then extracted into SDS sample buffer, run on a 10% gel, and stained with Coomassie Blue.

Hop Modulates Protein Folding
Hsp90, Hop and p23 bind to and stabilize distinct forms of hsp70. The binding of hsp70 (220 nM) to p23 (4.2 μM) and Hop was investigated in a two-step process with Hop and p23 incubated separately or together, under different nucleotide conditions. Lanes 1–5 measure the binding of hsp90 to p23. hsp90 was incubated at 30 °C for 30 min without nucleotide (lane 1), with 5 mM ADP (lane 2), 5 mM ATP (lane 3), ATP plus 2.6 μM Hop (lane 4), or with ATP at 30 °C and subsequent addition of Hop at 0 °C (lane 5). p23 was then added at 0 °C for 30 min, and complexes were recovered using antibody to p23. Lanes 6–10 measure the binding of hsp90 to Hop. hsp90 was incubated at 30 °C for 30 min without nucleotide (lane 6), with ADP (lane 7), with ATP (lane 8), or with ATP plus p23 (lane 9). The sample for lane 10 was treated identically to the sample in lane 9 but without the 30 °C incubation. 430 nM Hop was added at 0 °C after the 30 °C incubation of each sample, and complexes were recovered using antibody to Hop. Samples were then extracted into SDS sample buffer, run on a 10% gel, and stained with Coomassie Blue.

occurs significantly when Ydj is present to stimulate hsp70 to hydrolyze ATP. If Hop could stimulate ATP hydrolysis by hsp70, there would be no need for Ydj's presence to observe Hop binding to hsp70 in the presence of ATP. Thus, it appears that Hop is not acting as an ATPase stimulator or a nucleotide exchange factor for hsp70. The difference between our results and those of Gross and Hessefort (27) could possibly be due to different sources of Hop. For example, the bacterially expressed human HOP used here might be different or defective in some function, or the protein purified from rabbit reticulocyte lysate may contain contaminants. hsp40 proteins are frequent contaminants of purified proteins that could easily produce misleading results (38).

We considered the option that Hop's activity in luciferase refolding in the presence of hsp70 and Ydj was to bind multiple hsp70's, bringing them into close proximity where they could act more efficiently on the substrate. However, this does not seem to be the case since, in the absence of hsp90, Hop produces the greatest stimulation of refolding when added in relatively high concentrations (Fig. 3A), meaning that complexes containing multiple hsp70's would be few and small. Also, we have never observed more hsp70 than Hop in immune precipitations of these proteins. Hop's effect could also be explained as changing the oligomeric state of hsp70 which has recently been shown to alter its ability to bind substrate (42). Shifting the hsp70 population toward monomer form would be expected to increase its affinity for substrate and increase refolding efficiency. Our hsp70 preparations are at least 98% pure and consist only of monomers. However, the hsp70 state could change during the course of an experiment. Our results agree with observations that Ydj is able to cause hsp70 polymerization which lowers its affinity for substrates (42), but Hop does not shift the hsp70 population toward the monomer form either in the presence or absence of Ydj (not shown). On the other hand, Hop does suppress the inhibitory effect resulting at high hsp70 concentrations and broadens the operating range of hsp70. This suggests that it is preventing the occurrence of a non-productive state of hsp70.

Our study reveals some valuable information on the nature of the interaction between Hop and hsp90. Studies on the binding of p23 to hsp90 indicate that the conformational state of hsp90 is regulated by ATP and ADP (34), and an ATP-binding site near the amino terminus of hsp90 has been recently identified (43, 44). It is well established that Hop and hsp90 form complexes, but we show that Hop binds preferentially to the form of hsp90 that exists either in the absence of nucleotides or in the presence of ADP. The ATP-bound form of hsp90 that is capable of binding p23 is not associated with Hop. We also show that Hop binding has the functional effect of...
proteins, the refolding of luciferase is still not as efficient as
the other.

However, our results indicate that the role of Hop is not simply
a folding complex, as its name hsp organizing protein implies,
but that Hop modulates protein folding.

Under our present conditions using four purified chaperone
proteins, the refolding of luciferase is still not as efficient as
refolding in rabbit reticulocyte lysate. The purified system is
optimized for the amounts of proteins that produce the best
overall refolding. Additional factors are most likely required
for efficient refolding, and two of the most obvious candidates are
the hsp70-interacting protein, Hip (46, 47), and p23. An impor-
tant aspect of future research will be to incorporate these, and
perhaps other factors, into the chaperoning system and to
define their mechanisms of cooperative action.

Acknowledgments—We thank Bridget Stensgard and Nancy
McMahon for technical assistance, and M. Christine Charlesworth for
assistance in protein purification. SF9 cell growth, treatment, and
harvesting were conducted by Dean Edwards and Kurt Christenson
at the University of Colorado Cancer Center Tissue Core.

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