ducers in an intermediate state from which they can be readily activated.

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26. Because the growth rate of cells originating from a different strain background (S288C) was less affected by deletion of *CPR7*, the magnitude of the effect of a *cpr7*Δ mutation on Hsp90-dependent events may vary from strain to strain.
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28. Samples of bacterial extracts containing GST-Cpr6 (*4*), GST-Cpr7 (*4*), or nonchimeric GST were incubated in the presence of glutathione–Sepharose 4B beads (Pharmacia) for 1 hour at 4°C. The beads were then washed twice with phosphate-buffered saline, and equal amounts of beads and bound proteins (~5 μg) were transferred to new tubes. The binding reactions were completed by addition to each tube of 2× binding buffer (100 μl) (*4*), yeast cell crude lysate [16 μl (80 μl in lane 8) extracted from strain W303 as described (*5*)], and doubly distilled H_2O to a final volume of 200 μl. After incubation at 4°C for 1 hour, the beads were washed four times with 1× binding buffer, and bound proteins were eluted with 0.5× final concentration of glutathione elution buffer (*4*). Material from 10 μl of each eluate (from a total of 50 μl) was resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and analyzed as described in the figure legend.
29. A plasmid expressing yeast Hsp90 (pETy90) was transformed into λDE3 lysogens of *E. coli* strain BL21 and induced with isopropyl-β-D-thiogalactopyranoside. Lysate (40 μl) derived from these cells was added to fusion protein–bound glutathione beads prepared as described (*28*). The three additional *GST::CPR* fusions generated for these experiments included GST fused at the NH_4-terminus of Cpr1, a hybrid between GST and the TPR-containing COOH-terminal region of Cpr6 (amino acids 171 to 371, Cpr6 tail), and a fusion between GST and the analogous region of Cpr7 (amino acids 193 to 393, Cpr7 tail). The binding reactions were treated as described (*28*), and eluates were analyzed by SDS-PAGE. Immunoblot experiments verified the identity of Hsp90 found in association with the fusion proteins.
30. Cells were transformed with a GR expression plasmid, p2A/GRGZ, that also contains the *lacZ* gene under the control of GR elements (*8*). In the absence of the receptor, the *lacZ* reporter gene is expressed at very low levels (*14*). Mid-logarithmic phase cells grown in galactose-containing medium were treated with saturating amounts (20 μM) of deoxycorticosterone (DOC) for 1 hour at 25°C, and β-galactosidase activity was assessed as described (*8*). Cellular proteins from mid-logarithmic phase cells were separated on an SDS-polyacrylamide gel and transferred to an Immobilion-P membrane. Equal sample loading was confirmed by Coomassie blue staining before incubation with antibody specific for GR (*8*) or L3 proteins. Constitutive GR activity was measured without DOC (*8*).

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Chaperone Function of Hsp90-Associated Proteins

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The Hsp90 heat shock protein of eukaryotic cells regulates the activity of proteins involved in signal transduction pathways and may direct intracellular protein folding in general. Hsp90 performs at least part of its function in a complex with a specific set of partner proteins that include members of the prolyl isomerase family. The properties of the major components of the Hsp90 complex were examined through the use of in vitro protein folding assays. Two of the components, FKBP52 and p23, functioned as mechanistically distinct molecular chaperones. These results suggest the existence of a super-chaperone complex in the cytosol of eukaryotic cells.

The heat shock protein Hsp90 is expressed at high levels in the cytosol of eukaryotic cells (*1*) and functions as a general cytosolic chaperone under physiological (*2*, *3*) and heat shock conditions (*4*) in vitro. Unlike other heat shock proteins, it seems to perform at least part of its activity in complex with proteins of unknown function (*5*, *6*). Nine different components of the complex have been identified, including Hsp90, Hsp70, p60/Hop (Hsp90/Hsp70 organizing protein), p48 (Hip), Hdj1, p23, and any one of the three large immunophilins: FKBP52 (FK506 binding protein, p59, Hsp56, HBI), FKBP54, or cyclophilin 40 (Cyp40) (*6*–*8*). Most of these proteins were initially identified in mammals in complexes with steroid receptors. They are required to keep the receptors in a state activatable by steroids (*6*, *9*). However, it seems that these complexes have more general functions because they are conserved throughout eukaryotes (*10*).

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To analyze the functional properties of the Hsp90 partner proteins, we studied their action in in vitro protein folding and unfolding reactions. As an assay system, the thermal unfolding and aggregation of citrate synthase (CS) was used (*4*). The characterization of the unfolding pathway of CS allows the quantitative analysis of the number of intermediates formed in the unfolding process and the distribution between native and aggregated forms of CS (*4*).

One of the characteristic features of molecular chaperones is their ability to suppress the aggregation of proteins under stress conditions (*1*, *11*, *12*). Thus, we examined the ability of Hsp90 partner proteins and a number of unrelated proteins to suppress the thermal aggregation process of CS. Immunoglobulin G (IgG), lysozyme, or bovine serum albumin (BSA) (Fig. 1A) (*13*, *14*), as well as an isolated domain of Hsp90 (*14*), did not affect the aggregation process of CS. However, two components of the mature Hsp90 complex, FKBP52 and p23, specifically suppressed the aggregation of CS in a concentration-dependent manner (Fig. 1, A and B), similar to that for Hsp90 (*4*) and small Hsps (sHsps) (*13*, *15*).

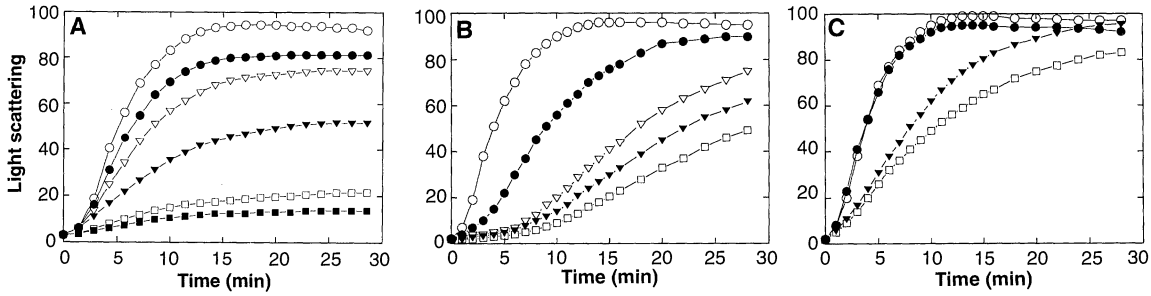

Fig. 1. FKBP52 and p23 suppress the aggregation of CS. The ability of the partner proteins to bind nonnative protein was examined by measuring the thermal aggregation of CS at 43°C. Proteins were purified as described (*28*). To determine thermal aggregation, we diluted 30 μM native CS 1:200 into 40 mM Hepes (pH 7.5), equilibrated to 43°C. Light scattering during thermal aggregation of CS (0.15 μM) was monitored (*4*) in the presence of (**A**) increasing concentrations of FKBP52 [(▽) 0.15 μM, (▼) 0.3 μM, (□) 0.6 μM, (■) 0.9 μM], (●) in the presence of 3 μM IgG, and (○) in the absence of additional components; (**B**) in the presence of increasing concentrations of p23 [(●) 0.15 μM, (▽) 0.75 μM, (▼) 1.5 μM, and (□) 2.25 μM], and (○) in the absence of additional components; and (**C**) in the presence of increasing Hop concentrations [(●) 0.15 μM, (▼) 1.5 μM, (□) 3.0 μM], and (○) in the absence of additional components.

Thus, FKBP52 and p23 were able to recognize and to bind efficiently and selectively to nonnative protein. In contrast, Hop (Fig. 1C) exerted a slight effect on CS aggregation only at very high protein concentrations. Of the three proteins tested, FKBP52 (Fig. 1A) was most efficient in suppressing aggregation. A molar ratio of 1:6 CS:FKBP52 caused almost complete suppression of aggregation. For p23 (Fig. 1B), a ratio of 1:8 CS:p23 resulted in half-maximal suppression of aggregation, and Hop (Fig. 1C) was inefficient, even at a ratio of 1:10 CS:Hop. The interaction of Hop with nonnative proteins seemed to be nonspecific because high concentrations of IgG (1:44 CS:IgG) also showed some effect on the aggregation of CS (compare Fig. 1, A and C).

There was no adenosine 5′-triphosphate (ATP) dependence observed for either FKBP52 and p23 (*16*). Thus, these proteins seem to belong to the class of ATP-independent chaperones such as sHsps (*13*), DnaJ (*17*), SecB (*18*), calnexin (*19*), and Hsp90 (*20*).

Having established that FKBP52, a member of the large immunophilin family of prolyl isomerases (*21, 22*), functioned as a molecular chaperone, we examined whether the prolyl isomerase activity of FKBP was the basis for its chaperone activity using immunosuppressive agents such as FK506 or rapamycin to inhibit its catalytic activity (*21–23*). The prolyl isomerase activity of FKBP52 was completely inhibited by rapamycin, both at 25°C and at 43°C (Fig 2A). However, FKBP52 was able to suppress the aggregation of CS with equal efficiency, both in the presence and absence of rapamycin (Fig. 2B), suggesting that the chaperone activity is independent of its prolyl isomerase activity. On the basis of sequence and hydrophobic cluster analysis, it has been proposed that FKBP52 can be divided into three domains (*24*), with the NH_2-terminal domain being responsible for the protein's prolyl isomerase activity (*25*). Thus, the chaperone activity of FKBP52 may be located in the other two domains.

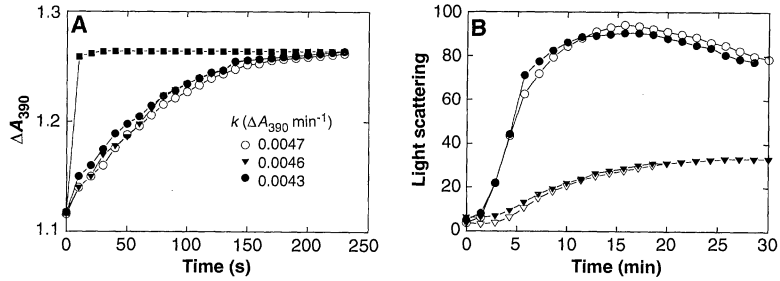

Fig. 2. The chaperone activity of FKBP52 is independent of its PPIase activity. (**A**) PPIase activity was measured in a coupled assay with chymotrypsin at 10°C (*29*) in the presence of (■) 0.9 μM FKBP52, (○) 0.9 μM FKBP52 incubated at 43°C for 30 min with rapamycin (50 μg/ml), (▼) 0.9 μM FKBP52 incubated at room temperature for 30 min with rapamycin (50 μg/ml), and (●) in the absence of additional components. The data were fitted to first-order rate equations with the use of Sigma Plot, and the apparent rate constants are shown in the inset. (**B**) Light scattering during the time course of thermal unfolding of CS (0.15 μM) in the presence of (▽) 0.9 μM FKBP52, and (▼) 0.9 μM FKBP52 incubated at room temperature for 30 min with rapamycin (50 μg/ml); (○) in the absence of additional components, and (●) with rapamycin (50 μg/ml).

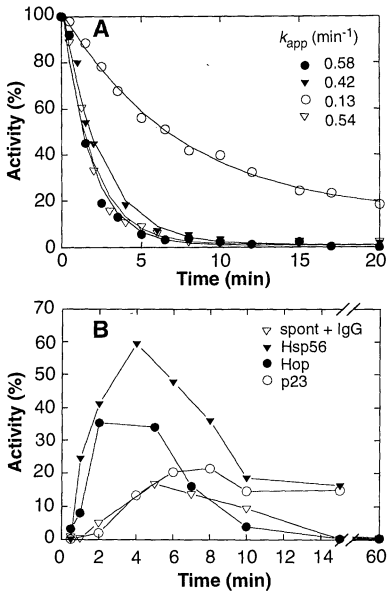

Fig. 3. Mechanism of chaperone action by Hsp90 partner proteins. (**A**) The effect of the partner proteins on the inactivation of CS activity at 43°C was measured (*4*). CS (0.15 μM) was incubated at 43°C in the presence of (▼) 0.9 μM FKBP52, (○) 0.3 μM p23, (●) 3 μM Hop, and (▽) 6.6 μM IgG. The solid lines represent single exponential functions with apparent rate constants as shown in the inset. (**B**) Analysis of the intermediates on the CS unfolding pathway was monitored in the presence of the isolated Hsp90 partner proteins. CS (0.15 μM) was incubated at 43°C in the presence of the various components, as indicated in (A). At the time points shown, reactivation of inactive CS molecules was initiated by the addition of oxaloacetic acid (OAA) (1 mM final concentration) and a shift to more permissive temperatures (25°C). After 60 min of incubation at 25°C, the activity of the reactivated samples was determined. The amounts of intermediates were calculated by subtracting the percentage of native species at the time of reactivation from the total amount of active species determined after the end of the reactivation process. The error in all cases was <5%.

Whereas the thermal aggregation assay gave an indication of the general chaperone function of each protein, analysis of the unfolding pathway of CS provides additional information about the underlying molecular mechanism (*4*). Hsp90 has been shown to slow the inactivation process of CS, which suggests that Hsp90 interacts transiently with the unfolding protein (*4*). On the other hand, sHsps, although able

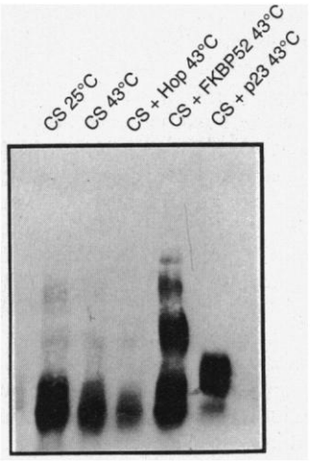

Fig. 4. Complexes between FKBP52 or p23 and nonnative CS. Complex formation between chaperone and CS after incubation for 5 min at 43°C was observed by cross-linking with glutaraldehyde (*30*) and subsequent immunoblotting with antibody to CS. The immunoblot was developed with ECL (Amersham).

to suppress aggregation effectively, appear to have little effect on the kinetics of inactivation (*15*) because they bind nonnative protein more stably and may require cooperation with other chaperones for release (*15*). An analysis of the inactivation of CS in the presence of the different Hsp90 partner proteins (Fig. 3A) showed that only p23 slowed the inactivation process, even at substoichiometric ratios. In contrast, FKBP52 and Hop showed little or no effect on the kinetics of the inactivation process. To further dissect the function of the individual partner proteins in protein folding, we examined the reactivation process of CS after thermal unfolding and quantitated the interaction of the partner proteins with unfolding intermediates of CS (Fig. 3B). In the presence of FKBP52, the amount of reactivatable intermediates of CS was increased during the first 5 min of the inactivation process to 60% compared with 18% in the absence of chaperone. FKBP52 maintained the amount of folding-competent intermediates at about 20% even after 15 min of inactivation when in the absence of chaperone all protein was irreversibly aggregated (Fig. 3B). An interaction of Hop with unfolding intermediates was observed only at high concentrations of Hop during the early phase of inactivation (Fig. 3B). Furthermore, the decrease in the amount of refoldable intermediates followed the same pattern as that of the control protein IgG. Thus, the interaction of Hop with nonnative proteins seemed to be nonspecific. These results correlate with the finding that Hop's function is primarily to mediate the association of Hsp90 and Hsp70 during the maturation of steroid receptors and kinases (*7*). In the presence of p23, the amount of folding-competent intermediates increased to just over 20% and remained at about 20% even after 15 min of inactivation (Fig. 3B). This result agreed well with the finding that p23 slowed inactivation (compare Fig. 3, A and B). Thus, p23 most likely binds transiently to early unfolding CS intermediates, in a manner similar to that for Hsp90 (*4*). However, p23 is more effective than Hsp90 in this process. In addition to the kinetic data, association between the chaperones FKBP52 or p23 and unfolding CS was directly demonstrated by cross-linking and subsequent immunoblotting with antibody to CS (Fig. 4). In the presence of p23, CS was completely shifted into a complex, whereas FKBP52 formed a complex with only a part of the unfolding protein (Fig. 4). No complex was observed with Hop. Thus, fundamental differences in the chaperone mechanism of the Hsp90 partner proteins exist, which suggest that FKBP52 and p23 have unique roles in assisting protein folding.

The large immunophilins have been shown to be catalysts only of prolyl isomerization (*21*, *22*). Now we show that, in addition, FKBP52 serves to chaperone protein folding and unfolding reactions by accumulating folding intermediates. This activity for large immunophilins has also been independently demonstrated for Cyp40, another immunophilin found in mature Hsp90 complexes (*26*). Thus, the large immunophilins in general may have acquired additional domains responsible for the chaperone function.

Although the prolyl isomerase activity of the immunophilins had already suggested their participation in the assisted folding process, the function of p23 has remained unknown until now (*5*, *6*). Because it is more potent than Hsp90 in stabilizing nonnative proteins, p23 seems to be one of the major players in the cytosolic super-chaperone machinery.

Taken together, our results suggest functional roles for the components of the Hsp90 chaperone complex. All the proteins associated with Hsp90, except for Hop, interact selectively and productively with nonnative proteins. However, their mode of action seems to be different, suggesting specific tasks in the complex. Given the abundance of this super-chaperone complex (*12*) and the lack of a cytosolic GroE equivalent in eukaryotes, the Hsp90 complex, in cooperation with the Hsp70 system, seems to be the major factor directing protein folding in the cytosol of eukaryotic cells.

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16. To investigate the ATP dependence of the chaperone properties of the Hsp90 partner proteins, we examined the effect of MgATP on their function during the thermal aggregation process of CS (*27*). In addition, we directly monitored the ability of these proteins to bind fluorescently labeled adenosine diphosphate analogs (*27*).
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28. All three recombinant proteins were purified as described below and their identity confirmed by immunoblotting and NH_2-terminal sequencing. Recombinant rabbit FKBP52 was purified by DE52, resource Q fast protein liquid chromatography (FPLC), hydroxylapatite, and size-exclusion chromatography. Recombinant human p23 was purified by DE52, $(NH_4)SO_4$ precipitation, and resource Q FPLC. Recombinant human Hop was purified by Q-Sepharose, hydroxylapatite, and size-exclusion chromatography. Mitochondrial citrate synthase was purchased from Boehringer Mannheim. Gel filtration and cross-linking data suggest that CS, Hop, and FKBP52 are dimeric and p23 is primarily tetrameric. All protein concentrations refer to the respective monomeric protein.
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