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Hsp90 Chaperone Activity Requires the Full-length Protein and Interaction among Its Multiple Domains*

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Hsp90 is an abundant and ubiquitous protein involved in a diverse array of cellular processes. Mechanistically we understand little of the apparently complex interactions of this molecular chaperone. Recently, progress has been made in assigning some of the known functions of hsp90, such as nucleotide binding and peptide binding, to particular domains within the protein. We used fragments of hsp90 and chimeric proteins containing functional domains from hsp90 or its mitochondrial homolog, TRAP1, to study the requirements for this protein in the folding of firefly luciferase as well as in the prevention of citrate synthase aggregation. In agreement with others who have found peptide binding and limited chaperone ability in fragments of hsp90, we see that multiple fragments from hsp90 can prevent the aggregation of thermally denatured citrate synthase, a measure of passive chaperoning activity. However, in contrast to these results, the luciferase folding assay was found to be much more demanding. Here, folding is mediated by hsp70 and hsp40, requires ATP, and thus is a measure of active chaperoning. Hsp90 and the co-chaperone, Hop, enhance this process. This hsp90 activity was only observed using full-length hsp90 indicating that the cooperation of multiple functional domains is essential for active, chaperone-mediated folding.

The importance of the 90-kDa heat shock protein (hsp90)[1] is clearly demonstrated by its abundance in all species with a remarkable 40% amino acid identity from *Escherichia coli* to humans (1, 2). Hsp90 is involved in numerous cellular processes, and deletion studies have shown that it is essential for viability in yeast (3, 4) and *Drosophila* (5). A number of cell-signaling proteins such as kinases and steroid receptors require hsp90 function to reach their active state within the cell (reviewed in Refs. 6 and 7). Several recent papers have conclusively established through biochemical and crystallographic studies that the amino-terminal domain of hsp90 binds ATP and ADP as well as geldanamycin (GA), a specific inhibitor of hsp90 function (8–11). Although the association of hsp90 with its co-chaperones is dependent on its nucleotide state (12–14), the mechanistic details of hsp90 action remain unclear.

In many of these processes, hsp90 does not act alone, but requires the aid of several co-chaperone proteins (6, 7). The interaction of hsp90 with its co-chaperones has been studied most extensively in the assembly of steroid receptor complexes (6, 15). In this process, hsp90 is found in two distinct complexes characterized by the presence of different sets of co-chaperone proteins. When it first enters steroid receptor complexes, hsp90 is associated with Hop and hsp70 (15, 16). Hop is a 60-kDa protein that is capable of binding both hsp70 and hsp90 when these proteins are in their ADP-bound state (12, 17). As the steroid receptor complex progresses toward the mature form capable of binding hormone, more hsp90 enters the complex while hsp70 and Hop levels diminish (15, 18). This mature form is also characterized by the appearance of the hsp90 co-chaperone p23, which interacts specifically with ATP-bound hsp90 (14), and one of three large immunophilins (15). Similar complexes between hsp90 and its co-chaperones are also found in the absence of any substrate protein, indicating that pre-assembled multiprotein complexes may act to chaperone a variety of substrate proteins (16, 19–21).

In addition to its role in the maturation of cell-signaling molecules, hsp90 has also been shown to play a role in more general protein folding. It is able to suppress the aggregation of denatured citrate synthase and β-galactosidase and maintain these enzymes in a refoldable state (22, 23). It also potentiates the refolding of firefly luciferase, *in vitro* and *in vivo*, by hsp70 and hsp40 (Ydj1) (12, 24, 25). A fragment containing the carboxyl-terminal 194 residues of hsp90 has been shown to convert MyoD1 to an active conformation *in vitro* (26). More recently, Scheibel *et al.* (27) and Young *et al.* (28) have reported that hsp90 contains two independent chaperone sites: one in the amino-terminal nucleotide-binding domain and the other in the carboxyl-terminal domain. Fragments containing these chaperone sites are able to bind to peptides with differing specificities and suppress the aggregation of unfolded proteins.

An assay for the chaperone-mediated refolding of thermally denatured firefly luciferase has been described previously (12, 25). In this system, the chaperones hsp70 and the yeast hsp40, Ydj1, are absolute requirements for the refolding process. Hsp90 can enhance refolding under many conditions, functioning in both a passive, ATP-independent manner, and in an active, ATP-dependent manner that can be augmented by Hop (12, 17, 25). These data, combined with recent reports regarding the abilities of two separate hsp90 fragments to act as chaperones prompted us to test a variety of hsp90 constructs

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[1] The abbreviations used are: hsp, heat shock protein; GA, geldanamycin; Hop, hsp organizing protein; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

for chaperone activity. In order to better define the functional domains of hsp90 involved in active and passive refolding processes, we tested a number of deletion mutants of hsp90 along with two chimeric forms of hsp90 and its mitochondrial homolog TRAP1 (29–31) in luciferase refolding as well as in the suppression of citrate synthase aggregation. The striking feature of the data is that while the suppression of citrate synthase aggregation can be accomplished by small fragments from within hsp90, essentially the entire hsp90 sequence is necessary for proper functioning in the refolding of luciferase.

EXPERIMENTAL PROCEDURES

Construction of Hsp90 Fragments and Chimeric Proteins—Wild-type, chicken hsp90, and the fragments of hsp90 shown in Fig. 1 were constructed using polymerase chain reaction to generate DNA fragments encoding the appropriate amino- and carboxyl-terminal protein sequences (32). These were subcloned into the pGEX expression vector (Amersham Pharmacia Biotech) such that the initiating methionine is in-frame with the GST. The sequences of the final plasmids were confirmed by automated DNA sequencing. The proteins were expressed in BL21(DE3) pLysS cells and purified by glutathione affinity chromatography followed by Mono Q chromatography.

The hsp90/TRAP1 chimeric proteins, ^{N}90-TRAP and ^{NC}90-TRAP, were constructed using polymerase chain reaction to generate DNA fragments encoding the appropriate amino- and carboxyl-terminal protein sequences. The primers h90b-85(*Nde*I) 5′-GATCGATCCATATGCCTGAGGAAGTGCACCATGGA-3′ and either h90b-726rev(*Eco*RV) 5′-GGTGATGGGATATCCTATGAACTGAGAATG-3′ (for ^{N}90-TRAP) or h90b-920rev(*Cla*I) 5′-TGATCGATGTATTTCTCTTTGATCTTCTTAGT-3′ (for ^{NC}90-TRAP) were used to generate amino-terminal hsp90-encoding DNA fragments. The primer TRAP1–803RV 5′-GATCGATCGATATCCCATCTACTTGAATGGAAGGCGGATGAAC-3′ or TRAP1–812Cla 5′-GTATCGATGGAAGGCGGATGAACACCTTGCA-3′ and SF-4 5′-AGTCAGTCGGATCCTTATCAGTGTCGCTCCAGGGCCTTGAC-3′ were used to generate carboxyl-terminal TRAP1-encoding DNA fragments. The NH_2-terminal hsp90 DNA fragments were digested with *Nde*I and *Eco*RV or *Nde*I and *Cla*I. The COOH-terminal TRAP1 DNA fragments were digested with *Eco*RV or *Cla*I and *Bam*HI. Each pair of fragments was then ligated into *Nde*I/*Bam*HI-digested pET9a. The sequences of the final plasmids were confirmed by automated DNA sequencing. ^{N}90-TRAP and ^{NC}90-TRAP proteins were produced in BL21(DE3) pLysS cells and purified as described previously for TRAP1 (30).

Purification of Hsp90—Human hsp90β was expressed in Sf9 cells using the system of Alnemri and Litwack (33), and purified as described previously (14). Cell lysates were fractionated by DEAE-cellulose column chromatography, followed by heparin-agarose and Mono Q chromatography. The preparation was greater than 99% pure as assessed by densitometry of SDS-PAGE gels. Protein concentration was determined by amino acid analysis.

Purification of Hsp70—Human hsp70 was expressed in Sf9 cells (33), and purified as described previously for avian hsp70 (25). Cell lysates were fractionated by DEAE-cellulose and ATP-agarose column chromatography. This was precipitated using ammonium sulfate (75% saturation), and the redissolved hsp70 was fractionated by 16/60 Superdex 200 FPLC. Only the monomer peak of hsp70 was used. The preparation was approximately 97% pure as assessed by densitometry of SDS-PAGE gels. Protein concentration was determined by amino acid analysis.

Purification of Hop—Human Hop expressed in bacteria was prepared essentially as described previously (34). Bacterial lysates were fractionated by DEAE-cellulose and hydroxylapatite column chromatography. Additional purification was achieved by fractionating the pool from hydroxylapatite on a Mono Q column (10/10, Amersham Pharmacia Biotech). The preparation was approximately 94% pure as assessed by densitometry of SDS-PAGE gels. Protein concentration was determined by amino acid analysis.

Purification of Ydj1—A bacterial expression system for Ydj1p was supplied by Dr. Avrom Caplan, and has been described previously (35). Bacterial lysates were fractionated by DEAE-cellulose and hydroxylapatite column chromatography. The preparation was approximately 80% pure as assessed by densitometry of SDS-PAGE gels. Protein concentration was determined by amino acid analysis.

Buffers and Materials—Tris buffer (TB) was 10 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 50 mM KCl, and 2 mM dithiothreitol. Stability buffer (SB) was 25 mM Tricine-HCl, pH 7.8, 8 mM $MgSO_4$, 0.1 mM EDTA, 10 mg/ml bovine serum albumin, 10% glycerol, and 0.25% Triton X-100. Geldanamycin was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute.

Aggregation of Citrate Synthase Assay—The thermal aggregation of citrate synthase molecules was measured as described previously (36). Citrate synthase (0.115 μM), in 40 mM HEPES, pH 7.5, was incubated at 43 °C to allow denaturation and aggregation to occur. The increase in optical density due to light scattering was measured at 336 nm to determine the extent of aggregation of the citrate synthase. These assays were carried out in the presence or absence of a variety of hsp90 constructs, at 0.575 μM each, five times the concentration of citrate synthase.

Luciferase Refolding Assay—Luciferase refolding assays were performed as described previously (25). Firefly luciferase, 100 nM in SB, was heat denatured at 40 °C for 15 min to ~0.2% of its original activity. This was diluted 10-fold into a refolding mixture containing purified chaperone proteins, 2 mM ATP, and an ATP-regenerating system in TB. The refolding mixture was incubated at 25 °C to promote folding, and at the indicated times following addition of denatured luciferase, aliquots were removed and luciferase activity was measured in a luminometer.

We performed all of the luciferase assays under conditions optimized to detect the hsp90 effect, which involved working at above optimal concentrations of hsp70. Because of this, misfolded proteins in the hsp90 fragment preparations could decrease the effective concentration of hsp70 and cause an increase in luciferase refolding by hsp70 and Ydj1 alone. This could easily be misinterpreted as a positive effect of the hsp90 preparation, and may account for the slight effects seen with some of the hsp90 fragment preparations. All assays were repeated several times and the results shown are typical of those results.

Protein Binding Assays—Hop binding to hsp90, TRAP1, ^{N}90-TRAP, and ^{NC}90-TRAP was assayed using 10 μg of each protein under conditions previously shown to be optimal for hsp90-Hop complex formation *in vitro* (25 mM Tris, pH 7.5, 50 mM KCl, 1 mM dithiothreitol, and 5 mM $MgCl_2$) (12). The protein mixtures were incubated at 30 °C for 30 min and added to antibody-protein A resins as indicated in the figure legends. The immunoprecipitations were incubated on ice for 1 h with occasional mixing and then washed four times with 1 ml of cold buffer. The proteins were eluted by boiling in SDS sample buffer, resolved by SDS-PAGE, and visualized by Coomassie Blue staining.

RESULTS

Fragments of Hsp90 Can Suppress Protein Aggregation—Previous studies have demonstrated the existence of two independent peptide-binding sites in hsp90 (27, 28). One of these sites is located in the amino-terminal nucleotide-binding domain of hsp90 and appears to be influenced by the highly charged region immediately following this domain (37). The other peptide-binding site is located near the carboxyl terminus. We used fragments of hsp90 fused to the dimeric protein, glutathione *S*-transferase (GST) to inhibit the aggregation of citrate synthase during thermal denaturation and confirm the existence of chaperone sites in both ends of hsp90. The hsp90 fragments used in this paper are shown in Fig. 1.

When denatured at 43 °C, citrate synthase aggregates; however, hsp90, at a molar ratio of 5:1, can prevent this aggregation to a large degree (see Fig. 6). The GST-wt construct also can effectively prevent citrate synthase aggregation, but GST alone lacks this ability (Fig. 2). In accordance with the results reported by others, a fragment from the amino terminus, GST-(1–573), and a fragment from the carboxyl terminus, GST-(446–728), are also able to suppress the aggregation of citrate synthase (Fig. 2). Additionally, fragments GST-(1–332), GST-(1–698), GST-(206–728), and GST-(287–728) were tested and each one is able to prevent citrate synthase from aggregating (summarized in Fig. 1). A fragment of hsp90 encoding residues 1–573 without GST was also expressed and like its partner, GST-(1–573), it is able to suppress citrate synthase aggregation, although it is less efficient than GST-(1–573) (Fig. 2). This may be due to the fact that GST-(1–573) is a dimer while 1–573 alone appears to be a monomer (not shown). In previous work by Young *et al.* (28) it can also be seen that hsp90 fragments are more efficient at preventing rhodanese aggregation when they

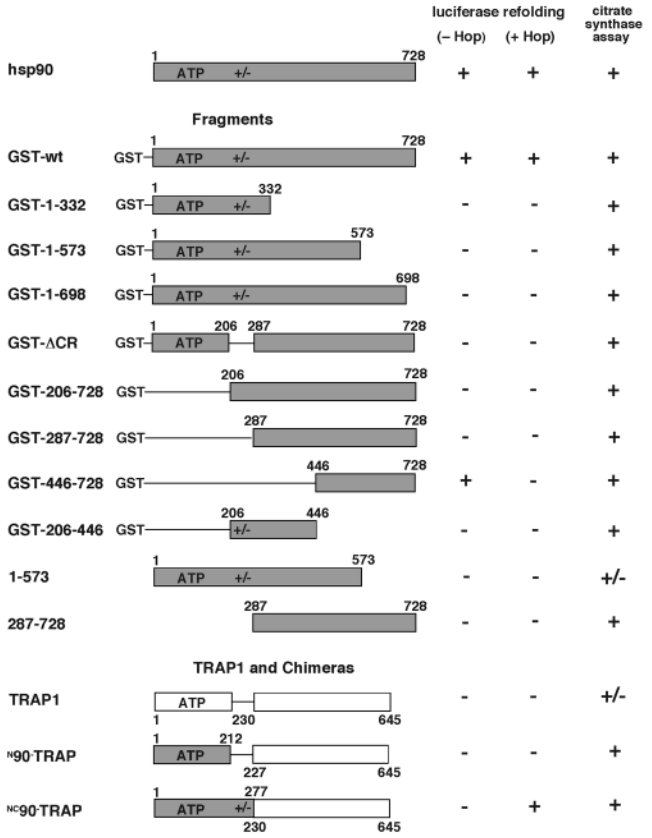

FIG. 1. **Constructs of hsp90 used in this study and summary of results.** This illustration shows the composition of hsp90, TRAP1, and the fragments and chimeras of hsp90 and TRAP1 used in this study. Boundaries are shown by the residue numbers *above* the *gray bars* for hsp90 residues, and *below* the *white bars* for TRAP1 residues. The ATP-binding domains (ATP) of hsp90 and TRAP1 and the charged region (±) of hsp90 are indicated where present. To the *right* of each construct is shown the results from luciferase refolding and prevention of citrate synthase aggregation assays. The activity of each construct is compared with the effect shown by hsp90 and categorized as full effect (+), reduced effect (+/−), or no effect (−).

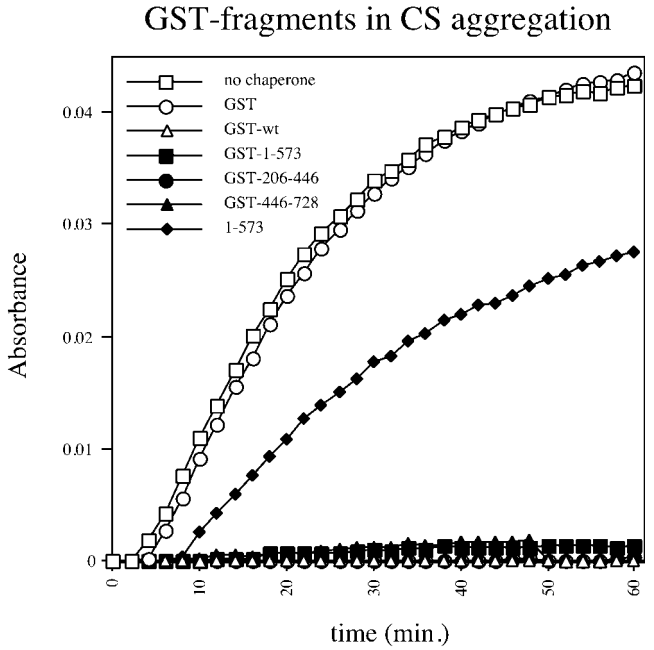

FIG. 2. **Fragments of hsp90 are able to prevent citrate synthase aggregation.** Aggregation of 0.115 μM citrate synthase was measured by the increase in absorbance due to light scattering during treatment at 43 °C for 1 h in the absence of any chaperone protein (□) and in the presence of five times the citrate synthase concentration (0.575 μM calculated as a monomer) of GST (○), GST-wt (Δ), GST-(1–573) (■), GST-(206–446) (●), GST-(446–728) (▲), and 1–573 (♦), and plotted as a function of time.

are fused to GST. Surprisingly, a fragment containing only a small portion of hsp90 from the central part of the molecule, GST-(206–446), is able to prevent the aggregation of thermally denatured citrate synthase as well (Fig. 2) suggesting this as an additional domain for peptide interaction.

Fragments of Hsp90 Are Unable to Assist Hsp70 and Ydj1 in the Refolding of Luciferase—We tested these same fragments for the ability to stimulate luciferase refolding by hsp70/Ydj1 in the absence and presence of Hop. When hsp90 is added to a refolding mixture containing hsp70 and Ydj1, it can substantially stimulate the refolding process (12, 17, 25). The chaperoning of hsp90 in luciferase refolding has an active component that is ATP-dependent, potentiated by Hop, inhibited by GA, and inhibited by point mutations that block ATP binding or hydrolysis (17, 25). The effect of hsp90 also has an ATP-independent component, termed passive chaperoning activity, that functions in the presence of GA and is unaffected by mutations in the ATP-binding site. We were interested in determining whether the fragments of hsp90 that can prevent aggregation also are able to participate in the refolding of firefly luciferase; a more stringent test of chaperone activity than simply binding to a protein to prevent its aggregation. We also wanted to determine whether the passive activity of hsp90 in luciferase refolding is the same activity we see in the ATP-independent citrate synthase assay, and whether we could identify discrete regions of hsp90 responsible for the active and passive chaperoning capacities.

The addition of a GST tag to wild type hsp90 does not alter the function of hsp90 in luciferase refolding (Figs. 3, *A* and *B*, 4, *A* and *B*). However, three GST fusion proteins containing amino-terminal fragments of hsp90, GST-(1–332), GST-(1–573), and GST-(1–698), are unable to stimulate luciferase refolding by hsp70 and Ydj1 either in the absence (Fig. 3*A*) or presence (Fig. 3*B*) of Hop despite the fact that each of these fragments is able to prevent citrate synthase aggregation. These hsp90 fragments are not aggregated and they have a functional ATP-binding domain that binds to ATP-Sepharose (results not shown). A fragment of hsp90 encoding residues 1–573 was also expressed and tested and, like its GST fusion protein, it shows no activity (not shown). Next, we tried an internal fragment from hsp90, GST-(206–446), and a fragment missing the charged region of hsp90 from residues 206–287, GST-ΔCR. Although both of these fragments can prevent citrate synthase aggregation, neither is able to stimulate luciferase refolding either in the absence (Fig. 4*A*) or presence (Fig. 4*B*) of Hop. Finally, we tried using carboxyl-terminal fragments of hsp90 in luciferase refolding, GST-(206–728) and GST-(287–728). Like the other fragments, these are able to prevent citrate synthase aggregation but unable to assist in luciferase refolding either in the absence (Fig. 5*A*) or in the presence (Fig. 5*B*) of Hop. These fragments do have the capacity to bind Hop (results not shown). Any inhibitory effect of GST can be ruled out because when the GST is cleaved from the amino terminus of GST-(287–728), the resulting fragment also has no activity (not shown). A comparison of the many hsp90 fragments in luciferase refolding (Figs. 3–5) *versus* in aggregation prevention (Fig. 2) shows a vast difference in the chaperone requirements for these two assays (Fig. 1).

GST-(446–728) Has a Limited Ability to Stimulate Luciferase Refolding—Of the many hsp90 fragments we tested for activity in luciferase refolding, only one shows any activity, GST-(446–728) (Fig. 5*A*). This activity is consistently seen, but only in the absence of Hop (Fig. 5*B*) even though this fragment

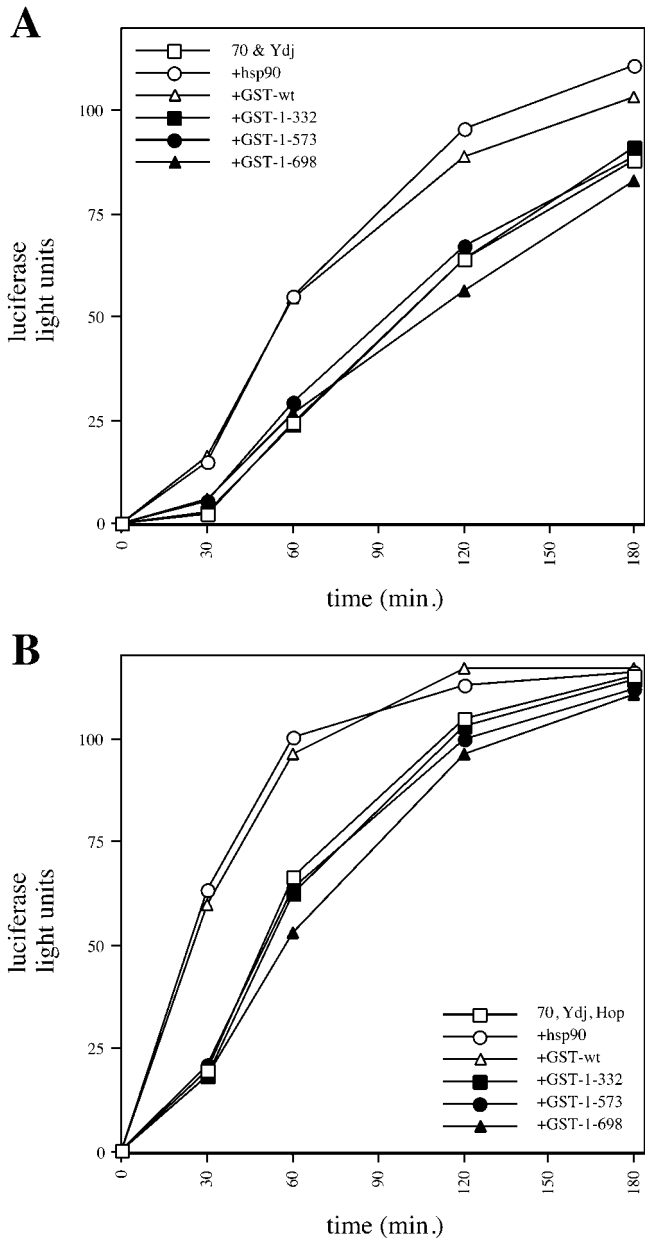

FIG. 3. **Amino-terminal fragments of hsp90 are unable to stimulate luciferase refolding.** Thermally denatured firefly luciferase (100 nM) was diluted 10-fold into a refolding mixture containing: *A,* hsp70 and Ydj1 (□), plus hsp90 (○), GST-wt (Δ), GST-(1–332) (■), GST-(1–573) (●), and GST-(1–698) (▲); *B,* same as in *A* but in the presence of Hop. The hsp70 concentration used was 1.33 μM, the Ydj1 concentration was 0.16 μM, the concentration of all hsp90 constructs was 0.5 μM, and the Hop concentration was 0.1 μM. Luciferase activity was measured at the indicated times and plotted.

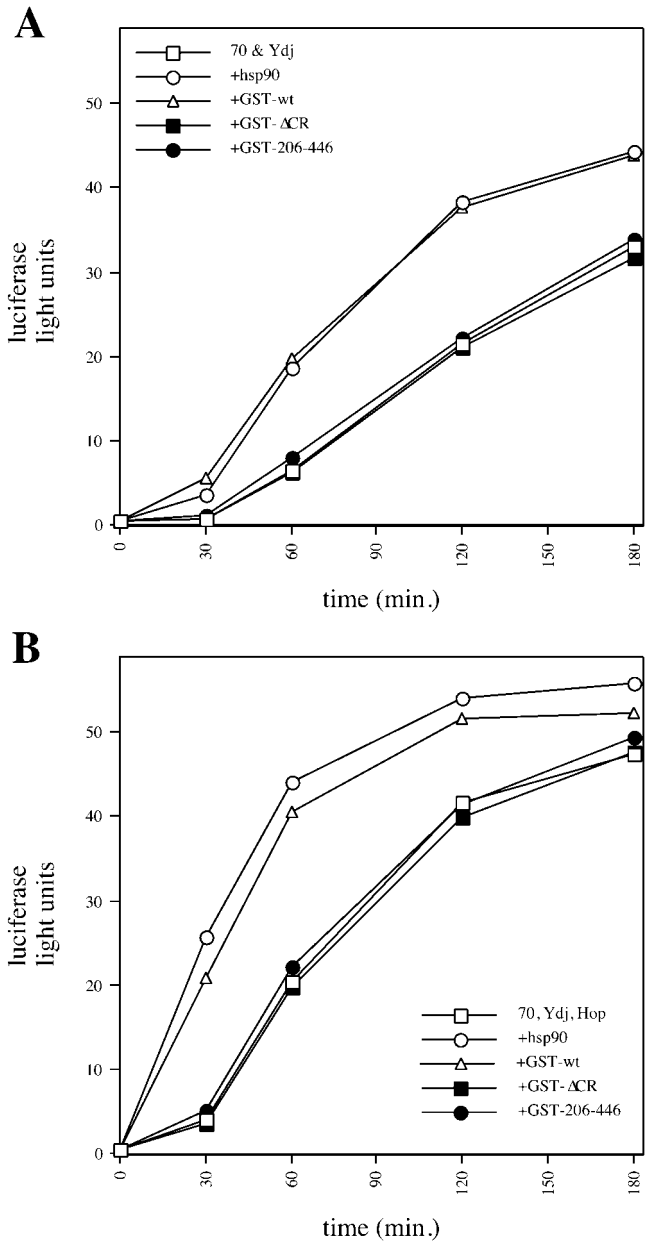

FIG. 4. **An internal fragment containing the charged domain of hsp90 and a fragment lacking the charged domain of hsp90 are unable to stimulate luciferase refolding.** Thermally denatured firefly luciferase (100 nM) was diluted 10-fold into a refolding mixture containing: *A,* hsp70 and Ydj1 (□), plus hsp90 (○), GST-wt (Δ), GST-ΔCR (■), and GST-(206–446) (●); *B,* same as in *A* but in the presence of Hop. The hsp70 concentration used was 1.33 μM, the Ydj1 concentration was 0.16 μM, the concentration of all hsp90 constructs was 0.5 μM, and the Hop concentration was 0.1 μM. Luciferase activity was measured at the indicated times and plotted.

can bind Hop (not shown). While hsp90s activity in luciferase refolding is partially sensitive to GA, the activity of GST-(446–728), like hsp70 and Ydj1 alone, is not inhibited by GA; thus it corresponds to the passive chaperone activity of hsp90 (Fig. 5*C*). The activity of this fragment is somewhat less than the passive activity of full-length hsp90 which remains in the presence of GA suggesting that the passive chaperone activity may not be fully contained within residues 446–728 (Fig. 5*C*). The amount of GST-(446–728) does not appear to be limiting because adding more causes no additional stimulation in refolding (Fig. 5*D*).

Chimeras of Hsp90 and TRAP1 Can Suppress Protein Aggregation—TRAP1, a mitochondrial member of the hsp90 family of chaperones has been recently shown to behave differently from hsp90 in a number of hsp90 functions (30). It cannot replace hsp90 in progesterone-receptor complexes and it is unable to bind the co-chaperones Hop and p23. We wanted to determine whether this homolog of hsp90 could substitute in luciferase refolding and in the prevention of citrate synthase aggregation. Furthermore, we made two chimeric proteins from hsp90 and TRAP1 by swapping key domains from hsp90 into TRAP1. Chimera ^{N}90-TRAP contains the NH_2-terminal 212-residue nucleotide-binding domain of hsp90 fused to residues 230 to the COOH terminus of TRAP1, and chimera ^{NC}90-TRAP is composed of the nucleotide-binding domain plus the next 65 residues from the highly charged domain of hsp90 (lacking in TRAP1), again fused to residues 230 to the COOH terminus of

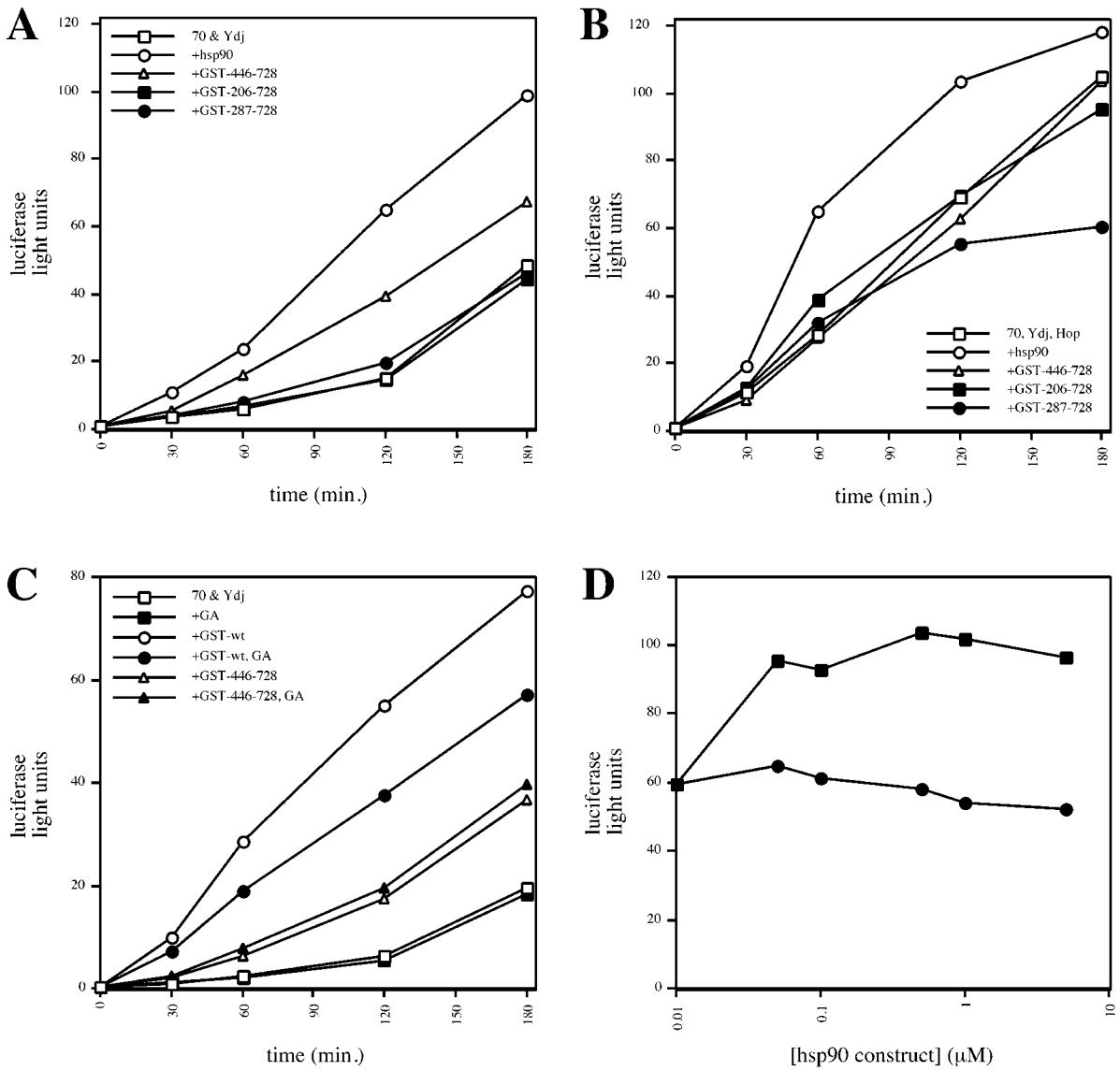

FIG. 5. **A carboxyl-terminal fragment shows some passive chaperone activity in the refolding of luciferase.** Thermally denatured firefly luciferase (100 nM) was diluted 10-fold into a refolding mixture containing: *A,* hsp70 and Ydj1 (□), plus hsp90 (○), GST-(446–728) (Δ), GST-(206–728) (■), and GST-(287–728) (●); *B,* same as in *A* but in the presence of Hop; *C,* hsp70 and Ydj1 (□), plus GA (■), GST-wt (○), GST-wt and GA (●), GST-(446–728) (Δ), and GST-(446–728) and GA (▲). The hsp70 concentration used was 1 μM, the Ydj1 concentration was 0.16 μM, the concentration of all hsp90 constructs was 0.5 μM, the Hop concentration was 0.1 μM, and the GA concentration was 10 μg/ml. Luciferase activity was measured at the indicated times and plotted. *D,* thermally denatured firefly luciferase (100 nM) was diluted 10-fold into a refolding mixture containing 1 μM hsp70, 0.16 μM Ydj1, and 0.1 μM Hop in the presence of 0, 0.05, 0.1, 0.5, 1, or 5 μM hsp90 (■) or GST-(446–728) (●). Luciferase activity was measured after 120 min of incubation for each concentration of hsp90 and GST-(446–728) and plotted *versus* the concentration of hsp90 or GST-(446–728).

TRAP1 (Fig. 1). We hoped these chimeric proteins would shed new light on the functions associated with some of hsp90s domains.

As seen in Fig. 6, hsp90 is able to suppress the aggregation of citrate synthase caused by treatment at 43 °C. When tested in this same assay, TRAP1 also is capable of suppressing aggregation, however, it does so with a much lower efficiency. It is important to note that despite the low efficiency of the interaction, a functional interaction between TRAP1 and denatured citrate synthase does occur, as not all proteins prevent aggregation in this assay (36). Both chimeric proteins are more efficient than TRAP1 at preventing aggregation, with ^{NC}90-TRAP being somewhat better than ^{N}90-TRAP, and both looking very similar to hsp90 (Fig. 6). Somehow the nucleotide-binding domain of hsp90 confers increased activity on TRAP1 in this assay, despite the fact that this is the region in which they have the greatest identity (30).

Hsp90-TRAP1 Chimera, ^{NC}90-TRAP, Is Able to Assist Hsp70, Ydj1, and Hop in the Refolding of Luciferase—We then tested TRAP1 and the chimeras, ^{N}90-TRAP and ^{NC}90-TRAP, for the ability to stimulate active and passive luciferase refolding by hsp70 and Ydj1 in the absence and presence of Hop. As seen above using fragments of hsp90, this assay is a more stringent test of chaperone function than is the prevention of protein aggregation. In the absence of Hop, neither TRAP1 nor the chimeras has any effect on luciferase refolding (Fig. 7*A*). When Hop is present in the refolding reaction, TRAP1 and ^{N}90-TRAP still show no activity, but ^{NC}90-TRAP displays a dramatic effect similar to the effect of hsp90 (Fig. 7*B*). The activity of this chimera corresponds to the active chaperoning of hsp90, as it is GA sensitive (not shown) and dependent upon cooperation with Hop (Fig. 7, *A* and *B*). Interestingly, ^{NC}90-TRAP always lags slightly behind hsp90 in luciferase refolding when comparing a range of concentrations (Fig. 7*B,* and results not shown).

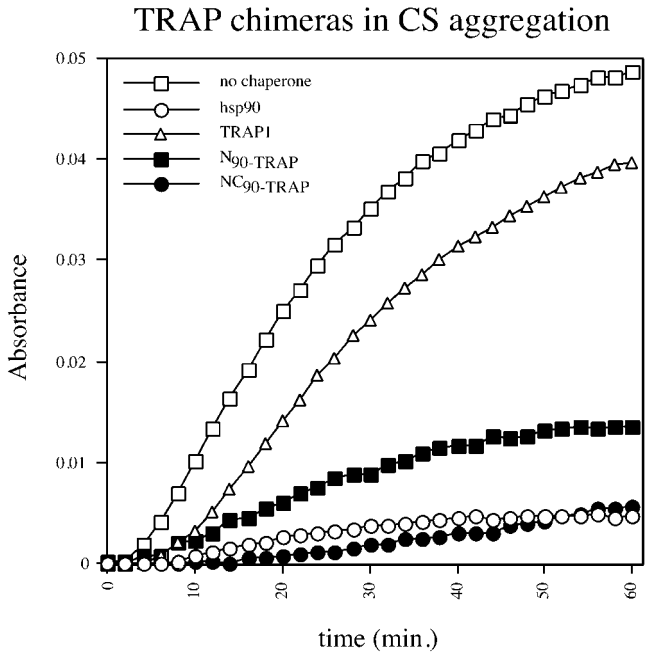

FIG. 6. **Hsp90·TRAP1 chimeras are able to prevent citrate synthase aggregation.** Aggregation of 0.115 μM citrate synthase was measured by the increase in absorbance due to light scattering during treatment at 43 °C for 1 h in the absence of any chaperone protein (□), and in the presence of five times the citrate synthase concentration (0.575 μM calculated as a monomer) of hsp90 (○), TRAP1 (Δ), ^{N}90-TRAP (■), and ^{NC}90-TRAP (●) and plotted as a function of time.

Chimera ^{NC}90-TRAP Requires Hop to Assist in Luciferase Folding, but Binding of Hop to ^{NC}90-TRAP Is Not Required—We have previously shown that TRAP1 does not bind to Hop (30), which is not surprising given the divergence of hsp90 and TRAP1 in the carboxyl-terminal, Hop-binding region. The carboxyl terminus of the hsp90-TRAP1 chimeras is derived from TRAP1, leading us to believe that they would be unable to bind to Hop. On the other hand, because Hop is required to see an effect of ^{NC}90-TRAP on luciferase refolding and because Hop's role in hsp70/hsp90-mediated chaperone processes is thought to reside in its ability to bring these two chaperone systems together, we reasoned that the two proteins must bind to each other. To test for a stable physical interaction between Hop and ^{NC}90-TRAP, we performed several immunoprecipitations using purified proteins. Hop was incubated with ^{N}90-TRAP, ^{NC}90-TRAP, hsp90, or TRAP1 followed by precipitation with an antibody to progesterone receptor, TRAP1, hsp90, or Hop (Fig. 8*A*). The results show that while hsp90 binds Hop to form a stable complex *in vitro* (*lanes 9* and *10*); TRAP1 (*lanes 12* and *13*), ^{N}90-TRAP (*lanes 2* and *3*), and ^{NC}90-TRAP (*lanes 5–7*) do not bind Hop.

We reasoned that a low affinity binding between Hop and ^{NC}90-TRAP might still exist and be essential for their role in refolding, although it could not be detected by immunoprecipitation. Thus we anticipated that Hop would be required in higher amounts when ^{NC}90-TRAP is used in refolding than when hsp90 is used. Using the luciferase refolding assay, we tested a range of Hop concentrations for their ability to stimulate refolding in the presence of hsp90 or ^{NC}90-TRAP (Fig. 8*B*). Concentration curves for Hop show no major increase in the Hop requirement when ^{NC}90-TRAP is substituted for hsp90 in refolding. This supports the result observed in immune precipitations.

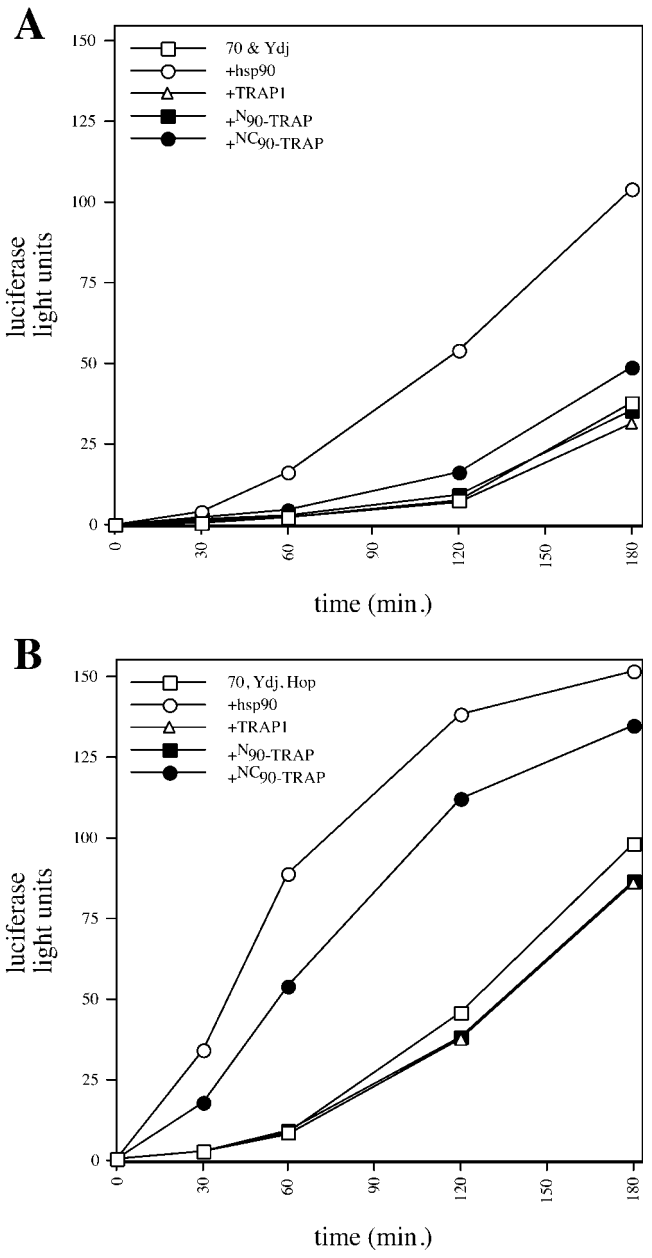

FIG. 7. **The charged domain of hsp90 is necessary for hsp90 chimera mediated stimulation of luciferase refolding.** Thermally denatured firefly luciferase (100 nM) was diluted 10-fold into a refolding mixture containing: *A,* hsp70 and Ydj1 (□), plus hsp90 (○), TRAP1 (Δ), ^{N}90-TRAP (■), and ^{NC}90-TRAP (●); *B,* same as in *A* but in the presence of Hop. The hsp70 concentration used was 1 μM, the Ydj1 concentration was 0.16 μM, the concentration of all hsp90 and TRAP1 constructs was 0.5 μM, and the Hop concentration was 0.1 μM. Luciferase activity was measured at the indicated times and plotted.

DISCUSSION

The past three years have brought great advances in our understanding of the important chaperone protein hsp90. We know the site at which nucleotides bind to hsp90 (9, 10, 17) and that GA shares this binding site (9, 39), giving us an understanding of its inhibitory effects on hsp90. We also know that ATP hydrolysis is essential to hsp90s activities (17, 40, 41), and that hsp90 is capable of binding substrate proteins in a simple, *in vitro* assay through both its amino- and carboxyl-terminal domains (27, 28). Information from the crystal structures of the amino terminus of hsp90 has been instrumental in establishing the binding sites for ATP and GA, but the chaperone function of hsp90 has been studied primarily using three functional assays: the ability to prevent aggregation of a denatured substrate (citrate synthase, rhodanese, insulin, and β-galactosidase), the ability to mediate the assembly of steroid receptor

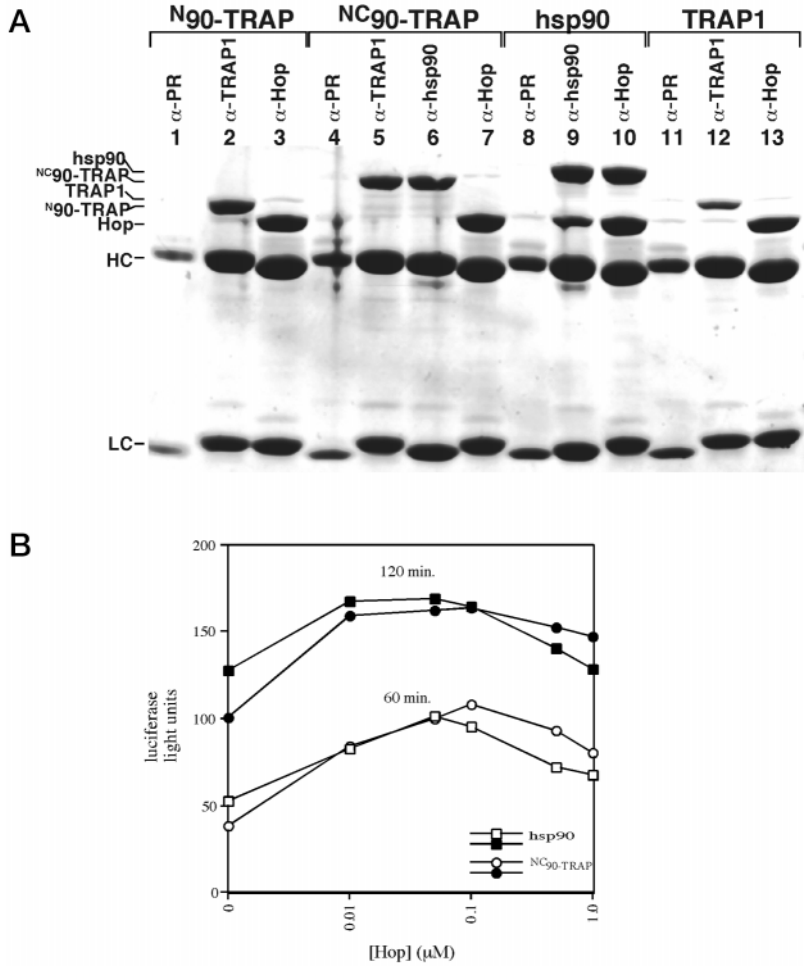

FIG. 8. **Hop binding to hsp90 chimera is not essential for hsp90-mediated stimulation of luciferase refolding.** *A,* purified Hop (10 μg) was incubated in the presence of 10 μg of ^{N}90-TRAP (*lanes 1–3*), ^{NC}90-TRAP (*lanes 4–7*), hsp90 (*lanes 8–10*), or TRAP1 (*lanes 11–13*) under optimal conditions for hsp90-Hop interaction. Antibody-protein A resins were added as indicated in the figure, and bound proteins were eluted, resolved by SDS-PAGE, and visualized by Coomassie Blue staining. *B,* thermally denatured firefly luciferase (100 nM) was diluted 10-fold into a refolding mixture containing 1 μM hsp70, 0.16 μM Ydj1, and 0.5 μM hsp90 (□ and ■) or ^{NC}90-TRAP (○ and ●) in the presence of increasing concentrations of Hop (0, 0.01, 0.05, 0.1, 0.5, and 1 μM). Luciferase activity was measured after 60 (□ and ○) and 120 (■ and ●) min of incubation for each concentration of Hop and plotted *versus* the concentration of Hop.

complexes in the presence of other chaperones, and the ability to stimulate the refolding of firefly luciferase in a mixture of chaperones. These assays each yield their own type of information, sometimes with conflicting results. To understand the information provided from each assay and use it properly to synthesize a conception of hsp90 function, some comparison of these assays is helpful.

The influence of hsp90 in the luciferase folding assay is complex. While full activity requires ATP and a functional nucleotide-binding domain of hsp90, partial activity is observed with ATP-binding mutants of hsp90 or in the presence of GA (17). However, this latter passive chaperoning activity is distinct from the ability of hsp90 to block aggregation of denatured proteins such as citrate synthase. If both assay types required the same activities, then fragments which bind peptides and prevent protein aggregation would also show passive chaperone activity in the refolding of luciferase. Our results show that, of all hsp90 constructs that were able to prevent citrate synthase aggregation, only GST-(446–728) showed any passive activity in luciferase refolding. Perhaps it should not be a surprise that the results from these two assays show little correlation. The protein aggregation assay is not dependent on energy input from ATP or on the presence of other chaperone proteins and both of these are requirements for the refolding of luciferase and the reconstitution of steroid-receptor complexes.

The fragments of hsp90 we used in this work have abilities similar to the fragments studied by others in preventing protein aggregation (27, 28). However, our fragments encompass different residues and could behave differently because one or the other is not capable of native-like folding (*i.e.* exposes an unnatural hydrophobic surface to the environment). An exposed hydrophobic surface caused by truncation of a protein could function, at least superficially, as a chaperone by binding to a peptide or a misfolded protein and preventing its self-association. This is supported by the observation that all of our fragments work in the citrate synthase assay although perhaps not all of them contain a physiological substrate-binding site. We do know that all of the constructs used in this study are soluble, readily purified, and not aggregated. Furthermore, all, except GST-(206–446), have been found to contain properly folded domains that can interact effectively with either ATP, Hop, or the hsp90 co-chaperone p23 (46). In a companion study,[2] we have tested the hsp90 constructs in Fig. 1 for their ability to chaperone the progesterone receptor. This activity was observed only with full-length hsp90 and GST-wt. Thus, these results are in agreement with those of the luciferase assay. While we believe that assaying for the prevention of protein aggregation can provide useful information on the nature of chaperone-substrate interactions, caution should be observed when extrapolating from this type of information to make predictions about more complex chaperone-mediated processes as there appears to be little correlation between the two.

Throughout much of this work, we use fragments of hsp90 fused to GST. It is important to note that the attachment of GST to the amino terminus of hsp90 does not have a detrimental effect on hsp90 function. When free in solution, hsp90 is dimerized in anti-parallel fashion through contacts near the COOH terminus, leaving the NH_2 termini distant from one another (42). GST-wt behaves similarly to hsp90 in preventing citrate synthase aggregation and in refolding firefly luciferase despite the fact that its amino termini are held together through the dimerization of GST. GST-wt also functions in the

assembly of progesterone receptor complexes and appears to interact normally with ATP, p23, and Hop (46). Since dimerization through GST enhances the passive chaperoning activity of fragment 1–573 (Fig. 2), it is possible that subunit interactions near the amino termini occur during the normal functioning of hsp90, as has been suggested previously based on structural studies (43, 44). The importance of the amino-terminal nucleotide-binding domain of hsp90 in active chaperoning has already been shown by its GA sensitivity and the detrimental effects of point mutations within the nucleotide binding pocket (17, 40, 41). Here, we again show its importance to active chaperoning as well as show its requirement in passive chaperone activity since both GST-(287–728) and GST-(206–728) lack the ability to refold luciferase. In contrast, the passive chaperoning activity displayed by GST-(446–728) in the luciferase assay seems to indicate that the isolated carboxyl terminus may be under some negative regulation by residues 206–446 in the absence of the nucleotide-binding domain. In the citrate synthase assay for the prevention of aggregation, GST-(446–728) is no more effective than other hsp90 fragments (not shown). Thus, the citrate synthase assay provides no indication of the functions needed for the passive chaperoning of luciferase.

Several of the constructs used in this work speak to the importance of the charged region of hsp90 to its chaperone functions, both active and passive. GST-ΔCR lacks the charged region of hsp90 and is no longer functional in supporting the refolding of luciferase. Chimera ^{N}90-TRAP, which has no charged region, is not active in luciferase refolding while ^{NC}90-TRAP, which has the charged region added, behaves similarly to hsp90 in active chaperoning. This is in agreement with recent work by Scheibel *et al.* (37) showing that the charged region is an important modulator of peptide binding to hsp90. However, GST-ΔCR functions very well in the prevention of citrate synthase aggregation. Therefore, the charged region of hsp90 is not absolutely required for this limited chaperone activity.

The importance of the carboxyl-terminal region to the chaperone function of hsp90 is highlighted by the inability of GST-(1–332), GST-(1–573), and GST-(1–698) to participate in any aspect of refolding. GST-(1–698) is only missing 30 residues from its carboxyl terminus. Thus, these few residues are essential to hsp90's chaperone activity, both passive and active, as seen in the refolding of luciferase. At least some of this chaperone activity is conserved in the carboxyl-terminal region of TRAP1. On its own, TRAP1 is not as efficient as hsp90 in preventing citrate synthase aggregation. However, this activity to suppress aggregation can be greatly improved by replacing the nucleotide-binding domain of TRAP1 with that from hsp90 (chimera ^{N}90-TRAP). In addition, the chimera ^{NC}90-TRAP has the additional property of retaining the active (ATP-dependent) chaperone capacity of hsp90. ^{NC}90-TRAP does not end in MEEVD nor does it stably interact with Hop. These data indicate that a yet undefined requirement for actively chaperoning is conserved in the carboxyl-terminal regions of both TRAP1 and hsp90.

We proposed in an earlier study (12) that the role of Hop in chaperone processes goes beyond simply bringing hsp70 and hsp90 into contact to unite these two powerful chaperone systems. Previous reports demonstrate that Hop can stimulate luciferase refolding in the presence of hsp70 and Ydj1 even when hsp90 is not present (12), that F5 antibody against Hop inhibits progesterone receptor maturation without disrupting hsp90·Hop·hsp70 complexes (16), and that the yeast Hop homolog, Sti1, can alter hsp90's ATPase activity (45). The data presented here also suggest a greater role for Hop than widely accepted. Chimera ^{NC}90-TRAP behaves like hsp90 in its ability to support active refolding in the presence of Hop. This chimera, however, does not appear to bind Hop. If Hop's mechanism of action is simply bringing together hsp70 and hsp90 as its name (hsp organizing potein) implies, then Hop should have no effect on refolding when chimera ^{NC}90-TRAP is used in place of hsp90. There are several possibilities for explaining these enigmatic results; an interaction between ^{NC}90-TRAP and Hop may be substrate-mediated, or the interaction may be through separate but interdependent effects on the substrate requiring no physical contact at all. While ^{NC}90-TRAP lacks the major region for binding Hop, Pearl and Prodromou (45) have suggested that Hop also interacts with a region in the ATP-binding domain and this secondary site might then mediate an interaction between ^{NC}90-TRAP and Hop. Additionally, it remains possible that the cooperative effect of Hop and hsp90 observed in luciferase refolding occurs through Hop's modulation of hsp70/Ydj1-mediated refolding (12).

Pearl and Prodromou (45) have shown that Hop inhibits the ATPase activity of hsp90 even though it binds near the C terminus of hsp90. An inhibitory effect of Hop is also suggested by our studies using GST-(446–728). This hsp90 fragment provides some chaperoning activity in the luciferase assay, but only in the absence of Hop. Thus, Hop may function to suppress certain activities of hsp90 until these activities are necessary.

The simple fact that our attempts at fragmenting hsp90 to define its functional domains failed to uncover isolated regions of chaperone function shows that the activity of this chaperone is a complex process involving multiple domains. While simple events such as binding interactions can be seen in isolated domains of hsp90, no domain is sufficient in itself to carry out the more complex tasks of hsp90 chaperone activity. The vital interactions between hsp90 domains may take a number of forms. Two possibilities we suggest are regulatory interactions between the ATP-binding domain and the conformation of a substrate-binding domain or the necessity of dimerization to the proper function of other domains.

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