In vitro evidence that hsp90 contains two independent chaperone sites

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Received 13 October 1997

Abstract Hsp90 is an abundant and constitutively expressed stress protein and molecular chaperone. Here we dissected human hsp90 into three major domains to identify the putative chaperone site at which hsp90 binds unfolded polypeptide. Surprisingly, both the N-terminal and the C-terminal domain of hsp90 prevent the aggregation of denatured polypeptides. The chaperone activity of the N-domain is inhibited by geldanamycin, a specific inhibitor of hsp90-mediated protein refolding. While both domains suppress protein aggregation, only the C-domain binds an antigenic peptide derived from VSV G. Based on these results, hsp90 may be the first chaperone to contain two independent chaperone sites with differential specificity.

Key words: Hsp90; Molecular chaperone; Geldanamycin; Human

1. Introduction

The cytosolic 90 kDa heat shock protein (hsp90) is a highly conserved, constitutively expressed stress protein in eukaryotes and prokaryotes. In eukaryotes, hsp90 is known to cooperate with hsp70/hsp40 and a number of additional factors (p60/Sti1, p23, p50/cdc37, the immunophilin FKBP52, and cyclophilin 40) in the folding of a range of signal transduction molecules, including steroid hormone receptors and various protooncogenic kinases [1–4]. In vitro experiments have shown that hsp90 can act as a general chaperone capable of preventing the aggregation of various unfolded polypeptides [5]. Refolding of these substrates then depends on the additional presence of hsp70, an hsp40 homologue and ATP [6]. As has been discovered recently, hsp90 is the cellular target of the benzoquinoid ansamycin drugs, such as geldanamycin (GA) [7], which had previously been thought to specifically inhibit the activity of tyrosine kinases. It is now clear that in live cells and cell lysates GA inhibits the refolding of these kinases and other substrates by hsp90, resulting in their efficient proteosome-mediated degradation [8].

Crystal structures of the ~25 kDa N-terminal domain of hsp90 have recently been reported [9,10]. Consistent with biochemical studies, this domain contains the unique site for the high affinity binding of GA [9]. An ATP molecule was also observed by crystallography to bind at the same site [10], suggesting that GA is an inhibitor of ATP binding to hsp90. While there is biochemical evidence for low affinity binding of ATP to hsp90 [11,12], an ATP requirement in the function of hsp90 has not yet been demonstrated. Models for the binding of peptides to the N-terminal domain of hsp90 have been proposed [9,13], but have so far not been supported by experimental evidence.

Here we dissected the human hsp90 molecule into independently folding domain structures with the goal of establishing structure-function relationships for this complex chaperone protein. Based on in vitro assays using purified hsp90 fragments and unfolded model substrate proteins, we show that hsp90 contains two independent sites with chaperone function, one located in the N-terminal domain and the other in the C-terminal domain, the latter of which has also been implicated in homodimerization of hsp90 [14,15]. The main results of this study are summarized in Fig. 1.

2. Materials and methods

2.1. Purification of proteins

Expression and purification of the three hsp90 domains N90, M90 and C90 have been previously reported [9]. To generate the glutathione-*S*-transferase (GST) fusion proteins, DNA sequences encoding N90, M90 and C90 were ligated into the pGEX-4T-3 vector (Pharmacia) such that no additional amino acids were encoded between the thrombin cleavage site and the hsp90 sequences. The fusion proteins were expressed and purified by glutathione affinity chromatography (GST Gene Fusion System, Pharmacia). The ΔN90 and ΔC90 polypeptides were expressed using the pET15b vector (Novagen), and purified by chromatography on Q-Sepharose FF or Mono-Q (Pharmacia) followed by HTP-agarose (BioRad). Hsp90 was purified from bovine brain [16]. p60/Sti1 was expressed as published [17] and purified by Q-Sepharose chromatography. Bovine rhodanese and firefly luciferase were purchased from Sigma, and rabbit reticulocyte lysate from Green Hectares (Wisconsin).

2.2. Measurement of protein aggregation

Purified rhodanese was denatured at a concentration of 100 μM in buffer containing 6 M guanidinium-Cl, 25 mM HEPES-KOH, pH 7.5. Denatured rhodanese was rapidly diluted to 0.5 μM into buffer containing 50 mM KOAc, 25 mM HEPES-KOH, pH 7.5 (buffer F0), plus different concentrations of chaperone proteins. The chaperone proteins were exchanged into buffer F0 by chromatography on NAP5 columns (Pharmacia) immediately before use. Aggregation of rhodanese was monitored over 10 min by the optical density at 320 nm, and the data were normalized using the aggregation in buffer alone as the standard [18]. Firefly luciferase was denatured at 50 μM under the same conditions as rhodanese, and the aggregation at 0.25 μM was monitored in the same manner as for rhodanese.

2.3. Geldanamycin treatment of proteins

Hsp90, GST-N90 and GST-C90 were incubated at 2 μM in buffer F0 containing 18 μM GA (Gibco) for 30 min at room temperature. GA was dissolved in dimethyl sulfoxide (DMSO), and the incubation mixture contained 1% DMSO. Mock treated protein was similarly incubated in buffer F0 with 1% DMSO. Repurification of the proteins was found to be necessary to remove trace amounts of DMSO that interfered with the aggregation assays (not shown). Hsp90 was repurified by binding to DE52 (Whatman) for 1 h, washing twice with 10 volumes of buffer F0, elution with 500 mM KOAc, 25 mM HEPES-KOH, pH 7.5 for 1 h, then exchanging into buffer F0 with NAP5 chromatography. The GST fusions were bound to glutathione-Sepharose (Pharmacia) for 1 h, washed twice with 10 volumes of buffer F0, cleaved with 30 units/ml thrombin (Pharmacia) for 4 h and the supernatant used in the aggregation assays. The supernatant con-

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PII S0014-5793(97)01363-X

tained the N90 and C90 domains at >95% purity as determined by SDS-PAGE, with no evidence of the full-length GST fusion proteins. At the concentration used, thrombin had no effect on rhodanese aggregation (not shown). The concentration of repurified protein was measured by Bradford assay (BioRad) and the binding to GA was tested as published [7].

2.4. Peptide binding

VSV8 peptide with the sequence RGYVYQGL [19] was synthesized using standard Fastmoc protocols on an ABI 430A synthesizer. *N*-ε-aminocaproic acid was coupled to the N-terminus, followed by fluorescein isothiocyanate (FITC). The peptide was cleaved using standard protocols, purified by C18 reverse phase HPLC and characterized by analytical HPLC and MALDI-TOF mass spectrometry. In binding assays, the peptide was incubated at 200 μM in buffer F0 containing 10 μM hsp90, N90 or C90 for 1 h at 30°C. The reactions were cooled to 4°C and separated on a Superose 6 column for hsp90, or a Superdex 75 column for N90 and C90. All chromatography was performed in buffer F0 at 4°C. One ml fractions were collected and the fluorescence intensity at 494 nm excitation and 518 nm emission was measured in a Fluorolog fluorescence spectrometer. Fractions were then analysed by trichloroacetic acid precipitation followed by SDS-PAGE.

2.5. Luciferase refolding

Luciferase was denatured in guanidinium-Cl as above and diluted to a concentration of 0.2 μM into buffer F0 containing 10 μM chaperone proteins. After 10 or 30 min incubation, an equal volume of reticulocyte lysate supplemented with 12 mM MgOAc and 10 mM ATP was added and the reaction incubated at 30°C for 90 min. Luciferase activity was monitored and normalized to the activity of native luciferase under identical final conditions as published [8].

3. Results and discussion

Human hsp90 was previously dissected by proteolysis into three domains, an N-terminal, a middle and a C-terminal domain [9] (see Fig. 1 for domain boundaries). To identify which of these contain the known chaperone activity of hsp90, proteins corresponding to each of the domains were purified after expression in *E. coli*, either separately (N90, M90 and C90) or as fusion proteins with glutathione-*S*-transferase (GST-N90, GST-M90 and GST-C90). In addition, proteins were purified that contained the combined N-terminal and middle domains (ΔC90), and the combined middle and C-terminal domains (ΔN90 (Fig. 1)). All bacterially expressed

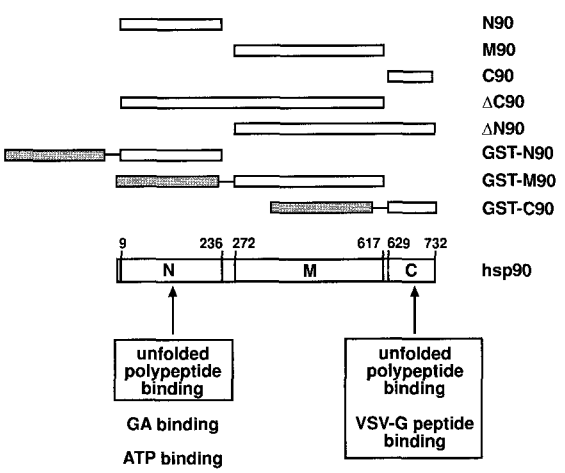

Fig. 1. Fragments of human hsp90 used in this study and summary of the main results. The hsp90 proteins are diagrammed as open bars, GST as gray bars. The domain boundaries used are shown as amino acid numbers.

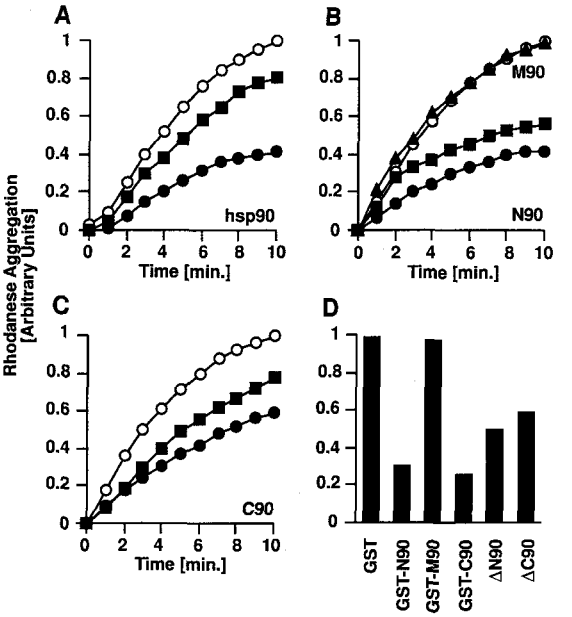

Fig. 2. N- and C-terminal domains of hsp90 prevent aggregation. A: Time course of 0.5 μM rhodanese aggregation in buffer (open circles), or buffer with 8 μM (filled circles) or 4 μM hsp90 (filled squares). B: Time course of 0.5 μM rhodanese aggregation in buffer (open circles), or buffer with 8 μM (filled circles) or 4 μM N90 (filled squares), or 8 μM M90 (filled triangles). C: Time course of 0.5 μM rhodanese aggregation in buffer (open circles), or buffer with 8 μM (filled circles) or 4 μM C90 (filled squares). D: Aggregation of 0.5 μM rhodanese after 10 min in buffer with 8 μM GST, GST-N90, GST-C90, GST-M90, ΔN90 or ΔC90.

proteins were fully soluble. As a control, full-length native hsp90 was purified from bovine brain.

The chaperone activity of hsp90 can be detected by the ability of purified hsp90 to prevent aggregation of unfolded polypeptide substrates such as citrate synthase [5] and rhodanese, which was used in this study. Both proteins have a pronounced tendency to aggregate upon dilution from the denaturant guanidinium-Cl (GdmCl) into a physiological buffer solution. This is easily monitored by the increase in optical density of the reactions due to light scattering. Hsp90 was capable of preventing this aggregation to a degree dependent on the concentration of chaperone in the reaction (Fig. 2A). Very similar results were obtained with the isolated N-terminal domain, N90, which also suppressed rhodanese aggregation in a concentration dependent manner (Fig. 2B). In contrast, the M90 protein showed no effect on rhodanese aggregation (Fig. 2B). Surprisingly, the C-terminal domain, C90, also had the capacity to suppress rhodanese aggregation and this activity also showed concentration dependence (Fig. 2C). Parallel results were obtained with the GST fusion proteins. While GST alone and GST-M90 were ineffective, GST-N90 and GST-C90 could both suppress rhodanese aggregation to a similar extent as the isolated domains (Fig. 2D). Chaperone activities of both GST-N90 and GST-C90 varied with the concentration of fusion protein used (data not shown). These results suggest that there are binding sites for unfolded polypeptides within both the N- and C-terminal domains of hsp90, and that these sites can operate in isolation.

To further corroborate these conclusions, we tested the constructs ΔN90 and ΔC90, combining middle and C-terminal domains and N-terminal and middle domains, respectively.

Both proteins were able to suppress rhodanese aggregation (Fig. 2D), supporting the notion that separate chaperone sites are present within the N- and C-terminal domains. Moreover, the chaperone sites are active while joined to the middle domain of hsp90, and therefore are likely to be active in the full-length hsp90 protein. This is particularly important with regard to the C-terminal domain, which has been implicated in the homodimerization of hsp90 [14,15]. Because ΔN90 behaves as a homodimer in gel filtration chromatography (not shown), the C-terminal chaperone activity is unlikely to arise from inappropriately exposed dimerization sequences of hsp90 monomers. The chaperone activities of hsp90, N90 and C90 were unaffected by adding purified p60/Sti1 (not shown), suggesting that neither of the chaperone sites are normally sequestered by p60/Sti1 or other tetratricopeptide repeat proteins.

The previous identification of the GA binding site within the N90 domain suggested that the drug might affect the chaperone activity of this domain. To address this question, hsp90, N90 and C90 were tested for the ability to suppress rhodanese aggregation after preincubation with a 9-fold excess of GA followed by repurification of the proteins (GA binds with high affinity and stays bound upon removal of free drug). Proteins subjected to a mock drug treatment were also tested for chaperone activity as a control. GA binding partially inhibited the activity of full-length hsp90 to suppress rhodanese aggregation (Fig. 3). Strikingly, the chaperone activity of N90 was almost completely inhibited by GA treatment (Fig. 3). Hsp90 and N90 repurified after drug treatment were unable to bind GA-affinity beads, in contrast to mock treated hsp90 and N90 (not shown), suggesting the effect on chaperone activity was due to drug remaining tightly bound to the proteins. As expected, the C90 protein, which is unable to bind GA [9], was only marginally affected by the GA treatment (Fig. 3). The partial inhibition by GA of hsp90 chaperone activity can be interpreted as evidence that at least in vitro, the N- and C-terminal domains contribute relatively independently to the chaperone activity of full-length hsp90, with one site sensitive to the drug and the other not.

We next addressed the question whether the two chaperone sites in hsp90 may have a differential structural specificity for target substrates. To this end, binding of a short synthetic peptide to N90 and C90 was analyzed. A fragment of vesicular stomatitis virus G protein, termed VSV8, with the sequence RGYVYQGL was chosen as a suitable substrate. VSV8 was originally identified in live cells as a specific substrate of GRP94, the endoplasmic reticulum homolog of hsp90 that is thought to bind antigenic peptides designated for presentation on MHC class I complexes [19–21]. GRP94 has 48% identity and over 70% similarity to the cytosolic form of hsp90, and can be subdivided into domain fragments corresponding to those of hsp90 (unpublished observations).

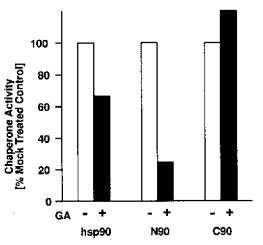

Fig. 3. Geldanamycin inhibits chaperone activity of the N-terminal domain of hsp90. Aggregation of 0.5 μM rhodanese was measured after 10 min in the presence of 7 μM hsp90, 4 μM N90 or 4 μM C90 treated with geldanamycin (GA, +) or subjected to a mock treatment (−). The decrease in rhodanese aggregation chaperone activity is expressed as a percentage of that observed using mock-treated chaperone (set to 100%).

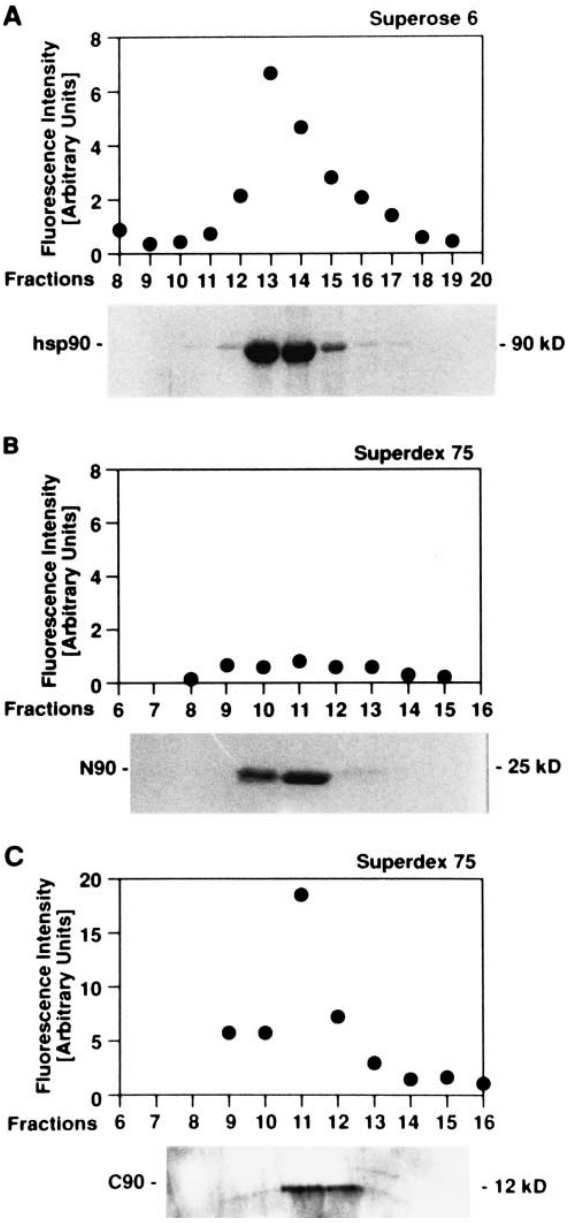

Fig. 4. VSV8 peptide binds to the C-terminal domain of hsp90. A: Hsp90 was incubated with a 20-fold excess of FITC-labelled peptide and the reaction separated by Superose 6 chromatography (see Section 2). FITC fluorescence of column fractions was measured and protein content determined by SDS-PAGE and coomassie blue staining. SDS-PAGE of each column fraction is shown below the corresponding fluorescence intensity. B: N90 incubated with peptide, and separated on a Superdex 75 column. C: C90 incubated with peptide and separated on a Superdex 75 column.

A 20-fold excess of synthetic peptide, labelled at the amino terminus with FITC to allow detection, was incubated with hsp90 and the mixture was separated by gel filtration chromatography. A peak in FITC fluorescence intensity was detected in the same fractions as hsp90, indicating that the pep-

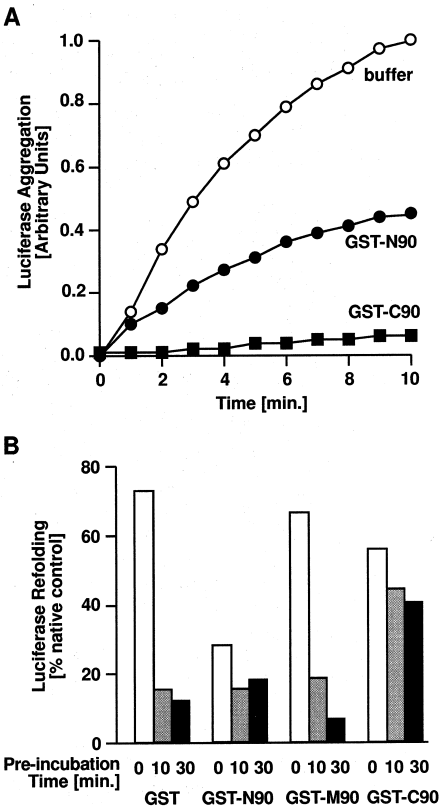

Fig. 5. The C-terminal domain of hsp90 maintains denatured luciferase in a folding-competent state. A: Time course of 0.25 μM luciferase aggregaton in buffer (open circles), or buffer containing 8 μM GST-N90 (filled circles) or GST-C90 (filled squares). B: Denatured luciferase was diluted to 0.2 μM into buffer containing 10 μM GST, GST-N90, GST-M90 or GST-C90 and preincubated for the stated time before refolding was initiated by the addition of reticulocyte lysate and Mg-ATP. The amount of refolded luciferase produced is presented as a percentage of equivalent native luciferase activity.

tide was indeed bound by hsp90 (Fig. 4A). A large peak corresponding to free peptide was detected at a much later elution volume, well resolved from the hsp90 peak (not shown). The efficiency of peptide binding by hsp90 was estimated at ~5%, similar to the <15% previously measured for GRP94 [22]. In contrast, when N90 was assayed, there was no detectable fluorescence in the column fractions containing the protein (Fig. 4B). However, column fractions containing C90 protein showed a very strong fluorescence signal (Fig. 4C). The higher fluorescence intensity observed from peptide bound to C90 compared with full-length hsp90 cannot be interpreted unambiguously as a higher level of binding, since the fluorescence signal may be quenched to some extent by the larger hsp90 protein. Nevertheless, the preferential binding of this peptide to C90 is initial evidence that the N- and C-terminal domains recognize different polypeptide sequences.

To further define differences between the N- and C-terminal chaperone sites of hsp90, the interaction with another unfolded polypeptide was examined. Firefly luciferase has been shown to interact with hsp90 in reticulocyte lysate and in live cells upon thermal denaturation [8]. As shown for rhodanese, GdmCl-denatured luciferase aggregated very rapidly upon dilution from denaturant. Both GST-N90 and GST-C90 were able to suppress luciferase aggregation in this assay (Fig. 5A). Consistent with the results obtained with rhodanese, GST alone or GST-M90 had no effect on luciferase aggregation (not shown). Because the N- and C-terminal hsp90 domains can act on unrelated polypeptides, such as rhodanese and luciferase, both domains may be generally involved in the function of hsp90 as a cellular chaperone.

Hsp90 has been shown to maintain unfolded β-galactosidase in a state competent for refolding by subsequently added hsp70 and hsp40 homolog [6]. The same activity of hsp90 has been reported for luciferase as the unfolded substrate [6], so a similar experiment was conducted to examine the activity of the N- and C-terminal domains in this refolding assay. GdmCl-denatured luciferase was diluted into physiological buffer containing either GST, GST-N90, GST-M90 or GST-C90 and the mixture was incubated for varying times before refolding was initiated by adding reticulocyte lysate and Mg-ATP (reticulocyte lysate contains hsp70 and hsp40 and is capable of refolding denatured luciferase). The final yield in activity of refolded luciferase was measured after 90 min. As expected, when denatured luciferase was diluted into a mixture containing GST, reticulocyte lysate and Mg-ATP without preincubation, a high level of luciferase refolding was achieved (Fig. 5B, GST, 0 min). In contrast, when unfolded enzyme was diluted into buffer containing GST followed by a preincubation of 10 or 30 min before the addition of lysate and Mg-ATP, very little active luciferase was produced (Fig. 5B, GST, 10 and 30 min). Similar results were obtained with GST-M90 (Fig. 5B, GST-M90). However, when the denatured luciferase was preincubated with GST-C90 for 10 or 30 min, significant levels of active luciferase were produced in the refolding reaction, closer to the refolding observed with no preincubation (Fig. 5B, GST-C90). Thus it appears that the C-terminal domain of hsp90, like full-length hsp90, can maintain an unfolded polypeptide in a folding-competent state, indicating that the chaperone activity of this domain in preventing aggregation can be utilized for productive folding.

Interestingly, GST-N90 inhibited luciferase refolding in the control reaction without preincubation (Fig. 5B, GST-N90, 0 min). Under the same conditions, GST alone or GST-M90 had no inhibitory effect (Fig. 5B, GST and GST-M90, 0 min). In other experiments, the isolated N90 also inhibited ATP-dependent refolding of luciferase in reticulocyte lysate, whereas hsp90 itself produced no inhibition (not shown). This effect may be related to the inability of hsp90 cofactors in releasing bound polypeptide from N90.

Based on the crystallographically determined structure of N90 [9,10,13], two very different models of peptide binding to this domain were proposed. Whereas in one model peptide is bound in or near the GA binding site [9], the other proposal suggests that the domain dimerizes including peptide in the domain interface [13]. Although these models cannot be distinguished on the basis of the data provided in this study, they suggest different mechanisms for the observed inhibition of N90 chaperone function by GA (Fig. 3). According to the former model, GA could act as a direct competitor for an unfolded polypeptide, while in the latter model GA would have to inhibit peptide binding allosterically. Such an allosteric mode of inhibition would be consistent with the view that GA functions as a competitive inhibitor of ATP [10]. However, addition of high concentrations of ATP (1 mM) did not inhibit the chaperone function of the N-terminal domain (not shown), which was significantly inhibited by concentrations of

GA as low as 18 μM followed by removal of excess drug (Fig. 3). It has been proposed that additional factors, such as p23, interact with hsp90 in an ATP-dependent manner [23]. Their effect on the chaperone activity of the N90 domain remains to be investigated.

The observed chaperone activity of C90 suggests a biological function beyond that of a simple dimerization domain. Since the C90 domain binds the antigenic VSV8 peptide, it is possible that the homologous domain of GRP94 contains the binding site for various antigenic peptide substrates. The ability of a C-terminal hsp90 fragment to enhance the activity of certain basic helix-loop-helix transcription factors in vitro [24,25] may also involve the chaperone activity of this domain.

The extent to which the two putative chaperone sites in hsp90 may be utilized physiologically remains to be established. Nevertheless, it is interesting to speculate on how the two sites may interact in a biological setting. For example, the in vivo effects of GA may be explained by its inhibitory effect on the chaperone activity of the N-terminal domain, which may result in the accumulation of hsp90 substrate polypeptides bound to the C-terminal domain. These questions will be the subject of future experiments.

Acknowledgements: The authors thank Alicia Russo and Mark Mayhew for their support in preparing purified hsp90 domains and VSV8 peptide. Peptide synthesis was performed by the Protein/DNA Technology Center of the Rockefeller University, New York. Part of this work was conducted at the Memorial Sloan-Kettering Cancer Center and was supported by the Howard Hughes Medical Institute. J.C.Y. is supported by the Medical Research Council of Canada. C.S. is a fellow of the German Center of Cancer Research.

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