Sti1 Is a Non-competitive Inhibitor of the Hsp90 ATPase

BINDING PREVENTS THE N-TERMINAL DIMERIZATION REACTION DURING THE ATPASE CYCLE*

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The molecular chaperone Hsp90 is known to be involved in the activation of key regulatory proteins such as kinases, steroid hormone receptors, and transcription factors in an ATP-dependent manner. During the chaperone cycle, Hsp90 has been found associated with the partner protein Hop/Sti1, which seems to be required for the progression of the cycle. However, little is known about its specific function. Here we have investigated the interaction of Sti1 from Saccharomyces cerevisiae with Hsp90 and its influence on the ATPase activity. We show that the inhibitory mechanism of Sti1 on the ATPase activity of Hsp90 is non-competitive. Sti1 binds to the N- and C-terminal part of Hsp90 and prevents the N-terminal dimerization reaction that is required for efficient ATP hydrolysis. The first 24 amino acids of Hsp90, a region shown previously to be important for the association of the N-terminal domains and stimulation of ATP hydrolysis, seems to be important for this interaction.

The molecular chaperone Hsp90 is known to be involved in the activation process of signal transduction molecules, such as kinases and transcription factors, among others (1–4). The essential in vivo function of Hsp90 in Saccharomyces cerevisiae involves ATP binding and hydrolysis of the nucleotide ATP (4, 5). ATP hydrolysis by Hsp90 is thought to involve conformational changes that lead to the transient association of the N-terminal domains of the dimeric chaperone Hsp90 (6, 7). These and additional structural rearrangements lead to the trapping of the ATP molecules inside the protein (8). In particular, the first 24 amino acids of Hsp90 are required to perform this “activation by dimerization” mechanism as they are thought to be swapped between the two N-terminal domains (9).

Studies performed in the context of the Hsp90-dependent steroid hormone receptors in higher eucaryotes have identified partner proteins, which are involved in the Hsp90-mediated activation process (10, 11). Transient association of these proteins with the Hsp90 chaperone machinery leads to a chaperone cycle that involves complexes of defined composition. These complexes are called “early complex,” “intermediate complex,” and “mature complex” respectively (12). The substrate proteins have to pass through these complexes to become activated. The early complex consists of the molecular chaperones Hsp70, Hsp40, and the co-chaperone Hop/Sti1. After association with Hsp90, the intermediate complex is formed. Here Hsp90 is primarily associated with Hop/Sti1, which serves as an adapter protein between Hsp70 and Hsp90 (10). In the mature complex, the proteins of the intermediate complex are exchanged for the proteins p23 and one of the large prolyl isomerases (FKBP51, FKBP52, or Cyp40) (13, 14). The details of this process, which ultimately leads to the activation of the substrate protein, are not known, but ATP hydrolysis by Hsp90 and Hsp70 is thought to play a crucial part in this process (15). The Hsp90 cycle seems to be evolutionary conserved since most of the Hsp90 partner proteins involved in this cycle are known to exist in yeast as well. In the yeast system, it had been shown that Hop/Sti1 acts as a potent inhibitor of the Hsp90 ATPase, and it has been speculated that this inhibition is achieved by blocking the ATP binding site (16). The polyisomerase Cpr6, a homologue of Cyp40, was able to reverse this inhibition, suggesting that the two proteins compete for the same binding site on Hsp90 (16). These sites, which involve TPR (tetratricopeptide repeat) domains, were shown to be at the C-terminal end of Hsp90. The crystal structure of the TPR domain in complex with a peptide and additional biochemical data suggest that the interaction between Hop and Hsp90 involves only the last 10 amino acids of Hsp90 (19, 20). Since the organization of the Hsp90 cycle critically depends on Sti1 as a central component, we decided to analyze the interaction between Sti1 and Hsp90 in detail. We show that the inhibition of Hsp90 by Sti1 is not achieved by blocking the access of nucleotide to its binding site but rather by restricting conformational changes of Hsp90 that are required for the hydrolysis reaction. Importantly, Sti1 binding involves multiple binding sites on Hsp90, including the first 24 amino acids.

MATERIALS AND METHODS

Materials—Radicicol was from Sigma. Geldanamycin was a kind gift of the Experimental Drug Division, NCI, National Institutes of Health, Bethesda, MD. All other chemicals were from Merck. Peptides were obtained from Dr. Susanne Modrow, University Regensburg, Regensburg, Germany.

Expression Constructs—Deletion mutants of yeast Hsp90 were generated using the plasmid pET28-HSP82, containing the full-length HSP82 gene of S. cerevisiae with an N-terminal His tag as a template (7). All PCR fragments were cloned into the pET28 vector, resulting in the constructs pET28-H8SP82, pET28b-D16-HSP82, and pET28b-D24-HSP82. The ΔMEEVD-mutant of Hsp90 has been generated using...
mutagenized primers. The other fragments mentioned in the text are as described in Richter et al. (7). pRSET-Cpr6 and pRSET-Sti1 are as described by Prodromou et al. (16).

Protein Expression and Purification—His-Hsp90 and its deletion mutants were expressed in the strain BL21 (DE3) cod + (Stratagene, La Jolla, CA) at 37 °C in LBteta, and induced with 1 mM isopropyl-thiogalacto-
actoside. Cells were lysed using a cell disruption system (Constant Systems, Warwick, UK). Protein purification was done according to the protocol described in Richter et al. (7). Proteins were stored in 40 mM HEPES, pH 7.5, 20 mM KC1 at concentrations of 1.5 mg/ml to 9 mg/ml at −80 °C. Mass spectrometry was used to verify the integrity and purity of the proteins. Purification of Sti1 and Cpr6 was achieved using essentially the same purification steps. Sti1 was stored at a protein concentration of 10 mg/ml, and Cpr6 was stored at a concentration of 11.5 mg/ml.

Stopped-flow Analysis—Stopped-flow measurements were performed with a HiTech SF-61 DX2 instrument in 40 mM HEPES, pH 7.5, 20 mM KC1, 5 mM MgCl2 using an ATP-nucleotide that is specifically modified at the γ-phosphate to carry the MABA-label. The excitation slit was set to 0.5 nm, the excitation wavelength was set to 298 nm to avoid nucleotide inner filter effects for tryptophan/MABA energy trans-
fer (FRET), and emission was detected through a cut-off-filter of 418
nm. The temperature was set to 25 °C unless indicated otherwise; concentrations indicated refer to the concentrations in the mixing
chamber.

Dissociation rate constants were measured directly by displacement of a preformed Hsp90(+)γ-MABA:ATP complex with excess unlabelled ligand. The observed kinetics followed single exponential equations. Association rate constants were derived from a series of experiments in which the concentration of [γ-32P]MABA:ATP was varied, whereas the concentration of protein was left unchanged. The individual time traces were analyzed with single exponential equations. Replots of these series of experiments with the observed rate constant (koff) as a function of ligand concentration followed straight lines, which is consistent with a simple, one-step binding mechanism. The rate constant for dissociation (koff) could be derived from the intercept and checked for consistency with the directly measured rate constant, whereas koff is represented by the slope. A replot of the observed amplitudes of the individual time traces versus concentration directly gives the dissociation constant (Kd), which can be compared with the one derived from the kinetic constants (koff/kon).

ATPase Activity—ATPase activities were measured using a regenerat-
ing ATPase assay as described by Ali et al. (21). The assays were performed in 120-μl cuvettes, and the reduction of NADH concentration was detected by the decrease of absorbance at 340 nm, using an Amer-
sham Biosciences 40/60 spectrophotometer. The temperature was set to 37 °C unless indicated otherwise. Assays were performed in 40 mM HEPES, pH 7.5, 5 mM MgCl2, and 2 mM ATP. KC1 was added at the concentrations indicated using a 1 M KC1 stock solution. Typical protein concentrations were 2.5 μM for Hsp90 and up to 20 μM for Sti1. To determine contaminating ATPase activities that could co-purify with Hsp90 or Sti1, radicicol, a specific inhibitor of the Hsp90 ATPase, was used at severalfold excess. The remaining ATPase activity in the pres-
ence of radicicol was interpreted to be background and was subtracted from the total activity.

Isothermal Titration Calorimetry—Isothermal titration calorimetry was performed using a Microcal VP-ITC instrument (Microcal Inc., Northampton, MA). For the binding of AMP-PNP to Hsp90 or Hsp90-Sti1 complexes, 4 mM AMP-PNP was used in the injection sy-
ringe. Protein concentrations were 15 μM Hsp90 to determine the bind-
ing constant of AMP-PNP to Hsp90. To analyze AMP-PNP binding to the complex of Hsp90 and Sti1, 32 μM Sti1 had been added to 15 μM Hsp90 prior to the isothermal titration calorimetry experiment. The buffer used in the syringe and the cell was 40 mM HEPES, pH 7.5, 150 mM KC1, 5 mM MgCl2 at 25 °C. As the binding constant for these conditions has been measured to be below 1 μM by SPR (22), this amount of Sti1 has been found to be sufficient to guarantee complete saturation with Sti1. 40 injections of ligand solution were made to fully saturate the protein in the cell. Data analysis was performed with the Origin software package (Microcal Inc.).

Surface Plasmon Resonance Spectroscopy—SPR analysis was carried out with a BiaCore X instrument (BiaCore, Uppsala, Sweden). Hsp90 was coupled to the surface of a CM5 sensor chip using amine coupling. The coupling has been performed according to the manufacturer's in-
structions. About 1200 resonance units of Hsp90 were coupled to flow cell 2 of the chip, whereas flow cell 1 was activated and blocked to obtain similar surfaces. First direct binding was used to obtain information about the response of the chip to Sti1 and Cpr6 at different concentra-

FIG. 1. Influence of KCl on the inhibition of Hsp90 by Sti1. Different concentrations of Sti1 were added to Hsp90 at various KCl concentrations, and the resulting ATPase activities were measured at 37 °C. The final concentrations of KCl were 80, 150, and 200 mM (○). The data points were analyzed using least square data analysis to obtain apparent binding constants for the Sti1:Hsp90 complexes.

FIG. 2. Influence of Sti1 on the Km value of ATP binding to Hsp90. ATPase activities were measured at varying ATP concentrations for preformed Hsp90-Sti1 complexes. Hsp90 concentrations were 4 μM, whereas for Sti1, the concentrations were 0, 8, and 16 μM (○, □). The activities were measured at 200 mM KCl in the ATPase buffer to prevent complete formation of complexes. The resulting activities were analyzed using least square data analysis.
Sti1 Is a Non-competitive Inhibitor of Hsp90—To investigate the mechanism of the Sti1-induced inhibition of the Hsp90 ATPase activity, we used steady-state kinetic approaches. Specifically, the analysis of the $K_d$ value of Hsp90 for ATP at different Sti1 concentrations should allow us to differentiate between the different modes of inhibition. As this methodology requires that the Hsp90 concentrations used are well below the dissociation constant of the Sti1-Hsp90 complex, we decided to use a KCl concentration of 200 mM for this approach. This implies a dissociation constant of about 10 μM for the Hsp90-Sti1 complex. The Hsp90 concentration used was 4 μM, and the Sti1 concentrations were 0, 8, and 16 μM, respectively. The $K_d$ values obtained for the ATP hydrolysis reaction were not affected by the presence of Sti1 (Fig. 2). However, the maximum velocity of the ATPase reaction was reduced. This behavior is usually interpreted as non-competitive inhibition.

Nucleotide Binding to the Sti1-Hsp90 Complex Is Not Affected by Sti1—Non-competitive inhibition implies that nucleotide binding is not affected in the Hsp90-Sti1 complex when compared with Hsp90 alone. We therefore performed isothermal titration calorimetry experiments in which we measured the binding of AMP-PNP to Hsp90 or to a preformed Hsp90-Sti1 complex. The titration curves were found to be nearly identical, both resulting in binding constants of about 30 μM (Fig. 3). These data clearly show that nucleotide binding to the Hsp90-Sti1 complex is still possible but leave the possibility that nucleotide binding might become rate-limiting in the case of the Sti1-Hsp90 complex. To test this, we determined the $K_m$ values of Sti1 between the different modes of inhibition. As this methodology requires a KCl concentration of 200 mM for this approach, this is a possible explanation for the weaker inhibitory effect, but more likely is the result of the weaker binding of Sti1 to Hsp90. The apparent binding constants of Sti1 and Hsp90 changed from below 1 μM at 80 mM KCl to 20 μM at 250 mM KCl.

Sti1 binding to Hsp90 involves C-terminal and N-terminal binding sites—The observation that the Sti1-mediated decrease in the turnover of the Hsp90 ATPase is the result of non-competitive inhibition raised questions about the mechanistic aspects of this interaction. Previously, it has been shown for human Hsp90 that Hop binds to the last 10 amino acids of this protein (20). Therefore we were interested to see whether these results could be extended to the yeast system. To address this question, we analyzed the interaction of Sti1 with Hsp90 by SPR spectroscopy. Hsp90 was immobilized on the surface of a CM5 sensor chip as described previously (22), and binding of Sti1 was detected based on the change in resonance units. Sti1 binding can be competed efficiently by the addition of Hsp90 to the injection solution. This allowed us to determine a dissociation constant of ~40 nM for the Hsp90-Sti1 complex, which is in agreement with previous data (22). As a control, we used the interaction between Hsp90 and Cpr6, which had been found to have a comparable affinity constant (22). Using this assay, we first analyzed an Hsp90 mutant lacking the last 5 amino acids, MEEVD. We could not detect binding of this mutant to Sti1 in the SPR assay, confirming the importance of this site for the interaction between TPR proteins and Hsp90 (Fig. 5A). To analyze whether this is the only site of interaction between the two proteins, we used peptides comprising either the last 7 or
Hsp90 and Hsp90/Sti1 complexes. 2.5 complex with 5/H9262/H18528/Hsp90/Sti1 complexes. 2.5 of MABA-ATP. A between the MABA-ATP and tryptophan residues near the binding site MABA-ATP bound to Hsp90 was recorded using FRET complexes. B plotting the observed rate constants against the MABA-ATP varying the MABA-ATP concentration upon binding to the proteins and result in binding of the peptides on the ATPase activity of Hsp90 has been observed A, B, Cpr6, also well above the binding constant of Cpr6 to Hsp90. For Cpr6, we could measure a binding constant of 5 or 10 Cpr6, indicating that the affinity of these peptides to Sti1 is much lower than that of Sti1 to Hsp90. The specific ATPase activity of wt-Hsp90 was used either alone (○) or in complex with 5 μM Sti1 (□). Data points were obtained by varying the MABA-ATP concentration upon binding to the proteins and plotting the observed rate constants against the MABA-ATP concentration.

The last 21 amino acids of Hsp90 as competitors in the SPR analysis of the interaction between Hsp90 and Sti1. These peptides were found to compete with Sti1 binding to Hsp90, albeit at about 1000-fold higher concentrations when compared with Sti1, indicating that the affinity of these peptides to Sti1 is much lower than that of Sti1 to Hsp90. For Cpr6, we could measure a binding constant of 5 or 10 μM, respectively, for the two peptides, also well above the binding constant of Cpr6 to Hsp90 (Fig. 5B). We further analyzed the interaction between these peptides and Sti1 using ATPase assays. No influence of the peptides on the ATPase activity of Hsp90 has been observed up to concentrations of 100 μM (data not shown), implying that binding of Sti1 to Hsp90 is probably 1000-fold stronger than binding of the peptides to Sti1. These data suggest that although the C-terminal amino acids play an important role in the interaction between Hsp90 and the TPR proteins Sti1 and Cpr6, additional binding sites are required for high affinity binding between the two proteins.

To address this issue, we performed competition experiments in which we used N-terminal and C-terminal truncated fragments of Hsp90 and determined dissociation constants for their interaction with Sti1 and Cpr6. This approach should allow us to detect differences in the binding of the two TPR proteins to Hsp90 (Fig. 6). Surprisingly, in the case of Sti1, the affinity for Hsp90 is decreased by a factor of about 8, when the N-terminal 24 amino acids of Hsp90 were missing (Fig. 6A, Table I). This effect was only observed for Sti1 and not for Cpr6, indicating a major difference in the binding of the two proteins (Fig. 6B, Table I). Clearly, these results suggest that Sti1 interacts with the most C-terminal part of Hsp90 and, at the same time, with the N-terminal domain. The cooperative nature of these interactions may be the reason that no interaction of Sti1 to each of the individual binding sites could be obtained.

Sti1 Has a Reduced Ability to Inhibit the ATPase Activity of Δ8-Hsp90—Based on the SPR data (Table I), Sti1 has been found to bind with nearly the same affinity to ΔMEEVD-Hsp90 as to wt-Hsp90. Compared with the other N-terminal deletion mutants, Δ8-Hsp90 is the only protein that still has an ability to hydrolyze ATP. The specific ATPase activity of Δ8-Hsp90 has been measured to be 1.7 min⁻¹ (9), and we therefore attempted to analyze the interaction between Δ8-Hsp90 and Sti1 using ATPase assays. Surprisingly, Sti1 was almost unable to inhibit the ATPase activity of this mutant under conditions used for the complete inhibition of Hsp90. An increase in the Sti1 concentration revealed that the effect seen is a result of weaker binding of Sti1 to Δ8-Hsp90 (Fig. 7). This disagrees with the SPR assays made so far (Table I). These data point to a mechanistic defect in the interaction between Sti1 and Δ8-Hsp90. This Hsp90-mutant has been described as forming N-terminal...
dimers with much higher affinity than wild type protein, but only in the presence of ATP (9). We therefore questioned the involvement of nucleotides in the observed interactions. To address this issue, we performed the SPR assay for Hsp90 and /H9004^8-Hsp90 in the absence and presence of nucleotides. Although for Hsp90 no influence could be observed, whether 2 mM ATP or no nucleotide was included in the running and injecting buffer (Fig. 8A), for /H9004^8-Hsp90, we observed a tighter binding to the nucleotide-free form (Fig. 8B). Thus, we conclude that the increased N-terminal dimerization in the presence of ATP is the result of the weaker binding of Sti1 to the ATP-bound form of /H9004^8-Hsp90 as compared with the nucleotide-free form of /H9004^8-Hsp90.

**Table I**

|         | Cpr6 | Sti1 |
|---------|------|------|
| Hsp90   | 41 nM ± 4 nM | 40 nM ± 4 nM |
| Δ8-Hsp90| 36 nM ± 3 nM | 111 nM ± 41 nM |
| Δ16-Hsp90| 18 nM ± 2 nM | 209 nM ± 55 nM |
| Δ24-Hsp90| 30 nM ± 8 nM | 571 nM ± 279 nM |
| 262C-Hsp90| 20 nM ± 3 nM | 675 nM ± 115 nM |
| 530C-Hsp90| 45 nM ± 6 nM | Not calculated |

**DISCUSSION**

Partner protein binding to Hsp90 is one of the characteristics of the chaperone cycle required for the proper maturation of the Hsp90 substrate proteins. Many Hsp90 partner proteins have been identified using steroid hormone receptors as natural substrates (23). These studies resulted in the identification of the prolyl-isomerases FKBp51, FKBp52, and Cyp40, as well as in the identification of Hop/Sti1 and p23/Sba1 (cf. Ref. 24). We know that a sequence of different defined Hsp90-containing complexes is required for the activation process of the substrate (11), but the function of the partner proteins in the chaperone cycle is still largely unknown. In the case of Hop/Sti1, the partner protein mediates the interaction between Hsp70 and Hsp90 in early complexes, and the S. cerevisiae homologue...
inhibits the ATPase activity of Hsp90 (16). For the reconstruction and deconvolution of the chaperone cycle, it is of importance to understand these interactions in detail, including the mode of inhibition of the ATPase activity of Hsp90.

Our data show that the inhibition, which had been reported previously to result from the steric hindrance of ATP binding to the N-terminal domain of Hsp90, is in fact the result of a non-competitive inhibition. Nucleotide binding to Hsp90 occurs normally, even if Hsp90 is in complex with Sti1. This observation prompted us to further investigate the binding properties of the two proteins. We identified a binding site for Sti1 in the first amino acids of yeast Hsp90, which agrees with data reported previously for hHsp90 and Hop (20). Here the C-terminal peptide was found to form a complex with the second TPR domain of Hop in which the peptide is completely surrounded by the α-helices of this domain. For the yeast protein, we were able to detect an additional Sti1 binding site in the N-terminal domain of Hsp90. Here, the deletion of the first 24 amino acids of Hsp90 resulted in an inhibition similar to that previously reported for H18528

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