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INTERACTIONS OF Hsp90 WITH HISTONES AND RELATED PEPTIDES

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Summary

The 90 kDa heat shock protein (Hsp90) induces the condensation of the chromatin structure [*Csermely, P., Kajtár, J., Hollósi, M., Oikarinen, J., and Somogyi, J. (1994) Biochem. Biophys. Res. Commun. 202, 1657-1663*]. In our present studies we used surface plasmon resonance measurements to demonstrate that Hsp90 binds histones H1, H2A, H2B, H3 and H4 with high affinity having dissociation constants in the submicromolar range. Strong binding of the C-terminal peptide of histone H1 containing the SPKK-motif and a pentaeicosa-peptide including the Hsp90 bipartite nuclear localization signal sequence was also observed. However, a lysine/arginine-rich peptide of casein, and the lysine-rich platelet factor 4 did not display a significant interaction with Hsp90. Histones and positively charged peptides modulated the Hsp90-associated kinase activity. Interactions between Hsp90, histones, and high mobility group (HMG) protein-derived peptides raise the possibility of the involvement of Hsp90 in chromatin reorganization during steroid action, mitosis, or after cellular stress.

Key Words: Hsp90, histone H1, HMG-protein, nuclear localization, signal, chromatin

Molecular chaperones help to maintain the conformational integrity of cellular proteins by governing their folding/unfolding during protein transport, assembly and degradation (1,2). The 90 kDa heat shock protein (Hsp90) is an abundant organizer of the cytosolic chaperone-complex (the "foldosome") helping *de novo* synthesized nuclear hormone receptors and several protein kinases to reach and preserve their unstable conformation in an "activation-competent" state (3-5). However, about 5 to 10 % of cellular Hsp90 is known to be localized to the cell nucleus and an additional fraction of Hsp90 translocates to the nucleus after a single or repeated heat shock (6-8). Several observations suggest that Hsp90 may also function as a nuclear chaperone during steroid action or after heat stress (9).

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Our earlier studies indicated that Hsp90 binds to histone H1 and induces the condensation of the chromatin structure (10,11). A detailed analysis of histone binding in the present report using surface plasmon resonance measurements indicates that though Hsp90 binds to histones with similarly high affinity, it displays significant differences in binding of other positively charged macromolecules. Histones and peptides having higher affinity for Hsp90 also modulate the Hsp90-associated kinase activity. Interactions between Hsp90, histones, and high mobility group (HMG) protein-derived peptides raise the possibility of the involvement of Hsp90 in chromatin reorganization during steroid action, mitosis, or after cellular stress.

Materials and methods

Chemicals — Histones H1, H2A, H2B, H3 and H4 were from Boehringer Mannheim (Germany). Geldanamycin was from GIBCO-BRL (Gaithersburg, MD, USA). [γ-^{32}P]-ATP (3000 Ci/mmol) was obtained from Amersham (Amersham, Buckinghamshire, UK). Peptides were synthesized using an Applied Biosystem 430A peptide synthesizer based on t-Boc chemistry, were purified by HPLC and their sequence was checked using an Applied Biosystems 477A Protein Sequencer and a 120A Analyzer. Peptide RRREEETEEE, was a kind gift of Dr. Steve Shoelson (Joslin Diabetes Center, Boston MA), casein basic peptide and platelet factor 4 were obtained from Iwaki Glass Co. (Chiba, Japan) and Peptide Institute Inc. (Osaka, Japan), respectively. The chemicals used for polyacrylamide gel electrophoresis, Bio-Scale Q FPLC and Econo-Pac HTP cartridges were from Bio-Rad (Richmond, CA, USA). Butyl-Sepharose 4B and DEAE Sepharose Fast flow were purchased from Pharmacia (Uppsala, Sweden). All other chemicals used were from Sigma Chemicals Co. (St. Louis, MO, USA).

Purification of Hsp90 — Hsp90 was purified from rat liver with consecutive chromatographies on Butyl-Sepharose 4B, DEAE-Sepharose Fast Flow, Econo-Pac HTP and Bio-Scale Q FPLC columns as described earlier (12). The purity of these Hsp90 preparations was more than 95 % as judged by silver staining of SDS polyacrylamide gels. Protein concentration was determined by the Bradford method.

Surface plasmon resonance (SPR) measurements — Rat Hsp90 was coupled to the SPR sensor chips (CM5, certified grade, Lot number: 02112) and SPR measurements were performed as described earlier (11). The usual change in the angle of the SPR minimum of the reflected light after coupling of Hsp90 was approximately 0.9 degrees, i.e. 9,000 resonance units (RU, range: 8500-9300) corresponding to 9 ng Hsp90/mm^2 sensor chip (11). With one coupling all measurements were made within 10 hours and the same sensor chip was used for maximum 3 days after opening. All the binding experiments were repeated with "mock-coupled" sensor chips, where in the coupling procedure Hsp90 was omitted. Binding curves (sensorgrams) were corrected by subtracting the electrostatic binding of histones and peptides to the matrix and the occasional changes in the refractive index after various additions. Special care was taken to reduce the latter phenomenon to the minimum throughout the experiments. Sensorgrams were analyzed by the BIA evaluation program 2.1 to obtain k_d values. The experimental data fit well the single exponential kinetic model used. Experiments were repeated at a flow rate of 20 µl/min to check the effect of possible rebinding. Rebinding and mass transfer limitation was also checked by the repetition of the experiments at lower levels of immobilized Hsp90 (3.5 ng/mm^2 sensor chip). The identical results showed that these phenomena did not occur under the conditions we used. Using the corresponding k_d values nonlinear curve fitting of the association phase of the corresponding sensorgram was carried out with the BIA evaluation program 2.1 to obtain k_a. K_d was calculated as k_d/k_a.

Measurement of Hsp90-associated protein kinase activity — Hsp90-associated kinase activity was determined in a 30 μl reaction medium containing 5 μg Hsp90, 0.1 mM [γ-^{32}P]ATP (7000-8000 cpm/pmol), 10 mM $MgCl_2$, or $CaCl_2$, as indicated, and 50 mM Hepes, pH 7.4 as described earlier (12). After an incubation of 20 minutes at 37 °C samples were subjected to SDS polyacrylamide gel electrophoresis and subsequent autoradiography. The amount of Hsp90-associated radioactivity was analyzed by an LKB Ultroscan laser densitometer and by liquid scintillation counting.

Determination of casein kinase II activity — Casein kinase II activity was determined using the specific peptide substrate, RRREEETEEE, as described earlier (13). Briefly, 20 μg of Hsp90 was mixed with 1 μg (0.7 mM) of RRREEETEEE peptide in 50 μl of reaction buffer containing 50 mM Hepes, pH 7.4, 0.1 mM [γ-^{32}P]ATP (1800 cpm/pmol), and 20 mM $MgCl_2$. Triplicate samples were incubated at 37 °C for 10 minutes. Incorporation of radioactive phosphate into the RRREEETEEE peptide was measured by its specific adsorption to P81 phosphocellulose papers followed by three repetitive washing steps in 75 mM phosphoric acid and liquid scintillation (13). Data were corrected to the absorption of residual [γ-^{32}P]ATP, and radioactivity of phosphorylated proteins by measuring identical samples without Hsp90 and the substrate peptide, respectively.

Results

Surface plasmon resonance studies — Our earlier studies indicated that Hsp90 binds to histone H1 and induces the condensation of the chromatin structure (10,11). Surface plasmon resonance measurements proved to be an efficient method to characterize the interactions of Hsp90 with other proteins, such as casein kinase II (13) and other interacting proteins (11). Encouraged by these findings we have utilized the high sensitivity of surface plasmon resonance to analyze Hsp90-histone interactions in detail. As it is shown in Table I, the dissociation constants of various histone/Hsp90 complexes are all in the submicromolar range indicating that the high affinity binding of histones to Hsp90 is a fairly general phenomenon.

TABLE I

Binding affinities of histones to Hsp90

Histones	$k_d \times 10^{-3}$ (s^{-1})	$k_a \times 10^3$ ($M^{-1}s^{-1}$)	K_d (nM)
Histone H1	0.9 ± 0.1	15.7 ± 1.1	57.3 ± 5.2
Histone H2A	9.6 ± 2.0	52.0 ± 5.1	185 ± 18
Histone H2B	7.7 ± 1.3	66.0 ± 8.5	117 ± 17
Histone H3	10.0 ± 3.0	32.0 ± 6.9	312 ± 47
Histone H4	2.9 ± 0.9	39.0 ± 11.1	74.4 ± 22.3

Hsp90/histone interactions were analyzed by surface plasmon resonance measurements as described in "Materials and Methods". Data are mean ± SD of three independent measurements.

To see the generality of Hsp90/basic peptide-protein interactions an SPR binding analysis has been performed with a variety of positively charged peptides. In contrast to the fairly general binding to histones, Hsp90 does not display a significant binding to poly-lysine, and two positively charged peptides, platelet factor 4 and a casein peptide (Table II, Fig. 1 and data not shown). On the other hand, Hsp90 shows a significant binding affinity for the carboxy-terminal peptide of histone H1 and for the bipartite nuclear localization signal of Hsp90, which is hidden in the native form of the protein (14,15; Fig. 1). Interestingly, binding of a glucocorticoid receptor derived-peptide containing a nuclear localization signal is much less pronounced (Fig. 1). Binding of the carboxy terminal histone H1 peptide is also compared to the binding of a 25-mer peptide from the basic C-terminal region of HMG14 displaying a similarity to histones (16), and shown to bind histone H3 (17), as well as a 25-mer peptide from HMG17 showing a homology with the γ-phosphate binding region of actin and other ATP-binding proteins (18) (Table II), respectively. Both the HMG14 and HMG17 peptides display a significantly lesser binding to Hsp90 than the histone H1 C-terminal peptide (Fig. 1).

TABLE II

Sequences of the peptides used in this study

Name	Sequence
histone H1 C-terminal peptide (▲)	KKPKKAAVKKSPKKAKKPAAAATKK
Hsp90 bipartite NLS (□)	EEEEKKDGDKKKKKKIKEKYIDEEE
HMG17 peptide (×)	DAGKEGNNPAENGDAKTDQAQKAEG
glucocorticoid receptor NLS (◆)	GMLEARKTKKKIKGIQQAT
HMG14 peptide (—)	KEEPKRRSARLSAKPPAKVEAKPKK
casein basic peptide (◆)	RPKHPIKHQGLPQEVLNENLL
platelet factor 4 (▲)	PLYKKIIKK

(the symbols after the peptides denote those of Figure 1.)

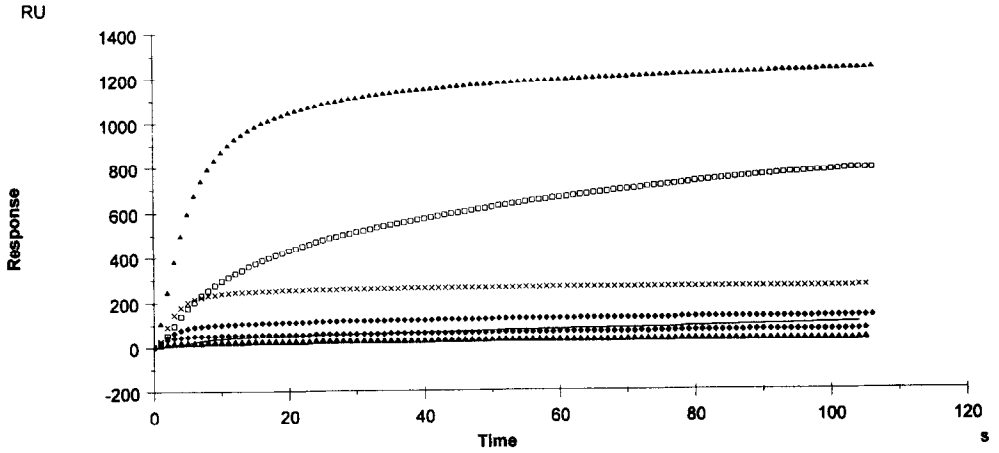

Fig. 1

Surface plasmon resonance (SPR) analysis of Hsp90-peptide complexes (at final concentrations of 100 μM, each). Surface plasmon resonance measurements were performed as described in "Materials and Methods". For the symbol explanations see Table II. Sensorgrams are representatives of three independent experiments.

Hsp90-associated kinase activity measurements — Comparison of the histone-induced activation of the Hsp90-associated kinase (12) indicates that histones H2A induces a 2-fold activation of the kinase, which is less than one third of the 7-fold activation by histone H1 (Fig. 2). On the contrary, histones H2B, H3 and H4 do not activate the Hsp90-associated kinase, which indicates that high-affinity binding *per se* is not enough to induce an activation of this Hsp90-related enzyme activity.

Since Hsp90 is known to associate with casein kinase II (12,13), it was important to analyze if the Hsp90-associated kinase measured in the present study corresponds to this kinase. Heparin-agarose affinity chromatography is a conventional method to remove casein kinase II (12). Comparing the Hsp90-associated kinase activity (measured by the Mg-dependent phosphorylation of Hsp90 itself) with the casein kinase II activity (measured by the phosphorylation of the specific substrate peptide, RRREEETEEE) a preferential loss of casein kinase II can be observed after heparin-agarose purification of Hsp90 (Fig. 3). As a further discriminatory feature of the Hsp90-associated kinase and casein kinase II, 5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole (DRB), a specific inhibitor of casein kinases (19,20) can significantly inhibit the residual casein kinase II of the Hsp90 preparation, while it hardly affects the Hsp90-associated kinase activity (41.2 % versus 12.3 % inhibition, respectively; Fig. 3). Similarly heparin, another inhibitor of casein kinase II (12), induces only a slight (14.1 ± 2.6 %) inhibition of the Hsp90-associated kinase. Thus our data strongly suggest that casein kinase II activity is only a small constituent of the activity observed. This assumption is further substantiated by the finding that histone-induced activation of Hsp90 persisted if examined with heparine-agarose purified Hsp90 (ref. 12 and data not shown). Activators and inhibitors of other Hsp90-associated kinases such as dsDNA (60 μg/ml), heme (0.2 mM), tyrphostin (0.1 mM) and H7 (0.1 mM) did not influence the effect of histones on Hsp90 phosphorylation (data not shown).

We were also interested to study if the positively charged peptides displaying a significant binding to Hsp90 also modulated the phosphorylation of Hsp90. Interestingly, only a few peptides such as the carboxy-terminal peptide of histone H1 and the similar amino-terminal peptide of HMG14 (16) induced significantly the Mg-dependent phosphorylation of Hsp90. Another group of peptides, such as the glucocorticoid receptor nuclear localization signal (NLS) peptide, the bipartite NLS-peptide of Hsp90, or a 25-mer peptide derived from HMG17 did not activate the Mg-dependent Hsp90-associated kinase activity (data not shown). Histone H1 was a potent activator of the Hsp90-associated kinase, while the histone H1 carboxy-terminal peptide induced the phosphorylation of Hsp90 only slightly, and even smaller effect was observed after the addition of the HMG14 peptide. The relative activating potential of these three basic substances correlated well with their binding efficiency to Hsp90 as shown by surface plasmon resonance data (cf. Figs. 1 and 4A). The carboxy-terminal histone H1 peptide-induced activation was biphasic, i.e. at higher concentration the activation was gradually reduced to the background level, which may indicate a multiple binding of the peptide to Hsp90 resulting in adverse effects on the Mg-dependent phosphorylation of Hsp90 (Fig. 4A). None of the peptides modulated the Ca-dependent autophosphorylation of Hsp90 (data not shown). We got essentially the same results, if re-examined some of the effects using heparine-agarose purified Hsp90 to remove the possible traces of casein kinase II (data not shown). Some of the peptides, such as the bipartite NLS-peptide of Hsp90, the glucocorticoid receptor NLS peptide, or the HMG17 peptide (for sequences see Table II) competed with both histone H1 and its carboxy-terminal peptide by diminishing their activation effect (Fig. 4B). The relative potency of the three peptides to inhibit the histone H1 or its carboxy-terminal peptide-induced activation was in agreement with our surface plasmon resonance data showing a binding efficiency in the order of Hsp90/NLS > HMG17 peptide > glucocorticoid NLS (cf. Figs. 1B and 4B).

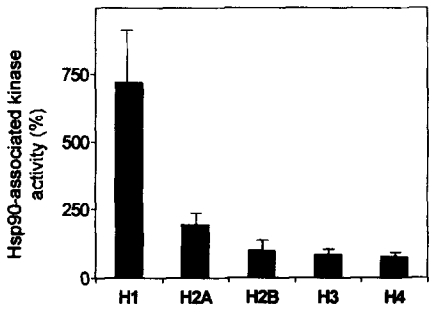

Fig. 2

Effect of histones to the Hsp90-associated protein kinase activity. Kinase activity measurements were performed in the presence of 10 mM $MgCl_2$ and 1 μM histones as described in "Materials and Methods". The initial, "100 %" kinase activity in the absence of histones corresponds to 3.5 pmol ^{32}P/min x mg Hsp90. Data are mean ± SD of three independent experiments.

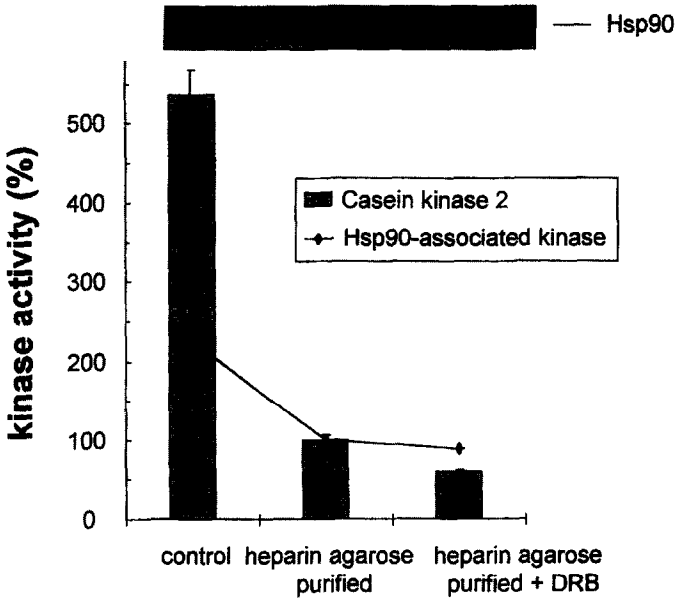

Fig. 3

Comparison of the Hsp90-associated kinase with casein kinase II. Highly purified rat liver Hsp90 was subjected to heparin-agarose affinity chromatography as described in ref. 12. Heparin-purified Hsp90 was treated with 100 μM 5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole (DRB), a specific inhibitor of casein kinases. Measurement of Hsp90-associated kinase and casein kinase II activities of control, heparin-agarose purified, and DRB-treated/heparin-purified Hsp90 was performed as described in "Materials and Methods". "100 %" Hsp90-associated kinase and casein kinase II activities correspond to 3.5 and 15.6 pmol ^{32}P/min x mg Hsp90, respectively. Data are mean ± SD of three independent experiments.

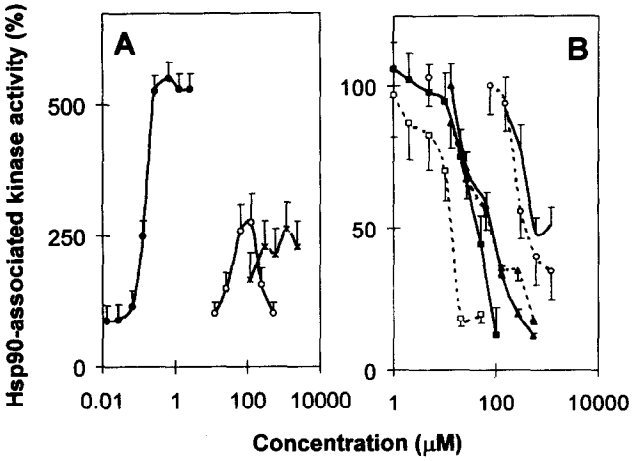

Fig. 4

Effect of histone H1 and related peptides on the Hsp90-associated protein kinase activity. Kinase activity measurements were performed in the presence of 2 μM Hsp90 and 10 mM $MgCl_2$ as described in "Materials and Methods". Panel A, Activation of Hsp90-associated kinase by histone H1 (filled circles), a carboxy-terminal peptide of histone H1 (open circles), and the functionally analogous amino-terminal peptide of HMG14 (asterisks). The initial, "100 %" kinase activity corresponds to 3.5 pmol ^{32}P/min x mg Hsp90. Panel B, Competition of the Hsp90 bipartide nuclear localization signal containing peptide (squares), the C-terminal HMG17 peptide (triangles), and the glucocorticoid receptor peptide containing a nuclear localization signal sequence (circles), with histone H1 (final concentration, 1 μM; filled symbols), and its carboxy-terminal peptide (final concentration, 100 μM; open symbols) as judged by their combined effect to the Hsp90-associated Mg-dependent kinase activity. The initial, "100 %" kinase activities in the presence of histone H1 and its C-terminal peptide correspond to 19.4 and 9.5 pmol ^{32}P/min x mg Hsp90, respectively. For the sequences of various peptides see Table II. Data are mean ± SD of three independent experiments.

Discussion

In the present report we have demonstrated that the high affinity interaction with Hsp90 is a fairly general feature of all histones tested. However, histone binding to Hsp90 is probably not an unspecific charge-charge interaction, since various positively charged macromolecules failed to bind to Hsp90 to the high extent observed by histones. Though a small amount of Hsp90 resides in the cell nucleus even in resting cells and a further amount of the protein is translocated to the nucleus after stress (5-9), steric hindrance imposed by the tight nucleosomal structure may prevent most of the observed histone-Hsp90 interactions *in vivo*. Our finding, that Hsp90 binds the C-

terminal tail of histone H1 is of particular interest, since this flanking histone segment is relatively accessible, and its SPKK motifs are involved in the regulation of chromatin function by their phosphorylation and acetylation (21-24). Hsp90 might interfere with these regulatory processes by masking the SPKK elements during stress or steroid action.

Hsp90 binds various nuclear localization signals (NLS) with relatively high efficiency. This finding is in agreement with earlier reports demonstrating the interaction of Hsp90 with NLS-sequences of the estrogen and glucocorticoid receptors (25,26) and casein kinase II (13). The NLS-specificity of Hsp90 is similar to that of Hsp70, which also binds to bipartite NLS-sequences at higher affinity than to an NLS containing only a single box of positively charged amino acids (27).

After our initial finding reporting a low-affinity binding of ATP and GTP to Hsp90 (12) the nucleotide binding of this chaperone became a bit controversial, since its comparison with Hsp70, which binds ATP at a much higher affinity, indicated significant differences in the nucleotide binding capabilities of the two proteins (28). However, recent data established a low affinity ATP-binding site in the N-terminus of Hsp90 (29) and by refined methods numerous evidences for the low affinity nucleotide binding of Hsp90 could be obtained (30,31). Autophosphorylation of Hsp90 occurs in the presence of Ca-ATP (12). The Hsp90-associated Mg-dependent kinase activity may be partially derived from the tightly associated casein kinase II content of Hsp90 (12,13). Since histone is known to activate casein kinase II (32,33), the effects of histone H1 and positively charged peptides on the Hsp90-associated Mg-dependent kinase activity might reflect their interaction with traces of casein kinase II in our Hsp90 preparation. However, the relatively unchanged effects after heat- or heparin-agarose treatment (data not shown), the preferential loss of casein kinase II activity after heparin-agarose purification of Hsp90, and the resistance of the Hsp90-associated kinase towards 5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole (DRB) and heparin, potent inhibitors of casein kinase II (19,20) all indicate that casein kinase II might be only partially responsible for the effects observed. Our earlier experiments also indicated that the Hsp90-associated Mg-dependent kinase activity can not be activated by polyamines (12), which also discriminates this activity from casein kinase II, which can be substantially activated by polyamines (32,33). The insensitivity for numerous other kinase activators and inhibitors makes unlikely the contribution of numerous known Hsp90-associated kinases (34) to the effects observed.

Histone H1 has the unique feature to activate the Hsp90-associated Mg-dependent kinase activity 6- to 7-fold, which is not shared by other histones despite their similarly high binding affinity towards Hsp90. The exact reason of this characteristic behavior of histone H1 is not known. However, it might be related to the fact that among all histones only histone H1 has been reported to possess an ATP-binding activity (23,35) and therefore it might participate in the ATP-presentation to Hsp90 or the ADP-removal from the chaperone activating the ATP-turnover of Hsp90.

The activation of the Hsp90-associated Mg-dependent kinase activity induced by histone H1, its C-terminal peptide, and the HMG14 N-terminal peptide corresponding to the DNA-binding domain of the protein (36) correlates well with the efficiency of their interactions with Hsp90, as demonstrated by surface plasmon resonance. Similarly, the relative SPR interaction of NLS and HMG17 peptides with Hsp90 also shows a good correlation with their relative concentrations inducing a half maximal inhibition of histone H1- and peptide-activated Hsp90-kinase. The HMG17 peptide is derived from the C-terminal region of HMG17 being responsible for transcriptional activation and interaction with histones. The competition of histone H1 and the

HMG17 peptide resembles to the competition of HMG17 with histones during the disruption of the higher order chromatin structure to enhance transcriptional processes (36-38).

Our data demonstrating that Hsp90 binds to the C-terminal peptide segment of histone H1 containing SPKK-motifs with high affinity, and that this interaction can be modulated by positively charged peptides containing nuclear localization signal sequences or derived from HMG17, raise the possibility that Hsp90 plays a role in the regulation of steroid-dependent transcriptional processes. Supporting this suggestion recent studies from *Donald DeFranco's lab* indicated that Hsp90 participates in the removal of glucocorticoid receptors from the chromatin (39). Interactions between Hsp90 and histones may reflect the involvement of Hsp90 in chromatin reorganization in various cellular processes.

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