Hsp90 chaperones wild-type p53 tumor suppressor protein

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Summary

Immortalized human fibroblasts were used to investigate the putative interactions of the Hsp90 molecular chaperone with the wild-type p53 tumor suppressor protein. We show that geldanamycin or radicicol, specific inhibitors of Hsp90, diminish specific wild-type p53 binding to the p21 promoter sequence. Consequently, these inhibitors decrease p21 mRNA levels, which leads to a reduction in cellular p21/Waf1 protein, known to induce cell cycle arrest. In control experiments, we show that neither geldanamycin nor radicicol affect p53 mRNA levels. A minor decrease in p53 protein level following the treatment of human fibroblasts with the inhibitors suggests the potential involvement of Hsp90 in the stabilization of wild-type p53. To support our in vivo findings, we used a reconstituted system with highly purified recombinant proteins to examine the effects of Hsp90 on wild-type p53 binding to the p21 promoter sequence. The human recombinant Hsp90 alpha isoform as well as bovine brain Hsp90 were purified to homogeneity. Both of these molecular chaperones displayed ATPase activity and the ability to refold heat-inactivated luciferase in a geldanamycin and radicicol sensitive manner, suggesting that post-translational modifications are not involved in the modulation of Hsp90 alpha activity. We show that the incubation of recombinant p53 at 37ºC decreases the level of its wild-type conformation and strongly inhibits the in vitro binding of p53 to the p21 promoter sequence. Interestingly, Hsp90 in an ATP-dependent manner can positively modulate p53 DNA binding after incubation at physiological temperature of 37ºC. Other recombinant human chaperones from Hsp70 and Hsp40 families were not able to efficiently substitute Hsp90 in this reaction. Consistent with our in vivo results, geldanamycin can suppress Hsp90 ability to regulate in vitro p53 DNA binding to the promoter sequence. In summary, the results presented in this paper state that chaperone activity of Hsp90 is important for the transcriptional activity of genotypically wild-type p53.
Introduction

The p53 tumor suppressor protein is a transcription factor, which regulates cellular response to stress, abnormal cell proliferation and DNA damage (1,2). More than 50% of human cancers possess mutated p53 gene (3), and the inactivation of p53 function leads to cell transformation (4,5). Most oncogenic p53 mutations are located in its DNA binding domain, inhibiting ability of this protein to initiate transcription (3). However, p53 may also be rendered inactive by other cellular events, such as the sequestration of wt p53* in the cytoplasm, due to increased nuclear export (6) or the association of p53 to cytoplasmic proteins (7,8). Recently it was shown that p53 is transported in HEK cells to the nucleus along microtubular tracks by cytoplasmic dynein and the Hsp90 molecular chaperone (9), which is similar to the mechanism described for rapid ligand-induced movement of the glucocorticoid receptor to the nucleus (10,11). Processes that inhibit p53 nuclear transport and proteolysis also lead to the inactivation of p53 protein (12,13).

In the response to various stresses, such as ionizing radiation, UV and hypoxia, p53 is activated, stabilized and imported into the nucleus, where it promotes transcription of several genes whose products induce cell cycle arrest, DNA repair or apoptosis (14). In a non-stress situation, the level of p53 in the cells is mainly regulated at the posttranslational level by MDM2 (12,15-17).

* AMP-PNP, Adenylyl-imidodiphosphate; ChIP, chromatin immunoprecipitation; CPT, camptothecin; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; GA, geldanamycin; Hsp90 bov, bovine brain Hsp90; Hsp90α, Hsp90 alpha isoform; mut p53, p53 in mutant conformation; R, radicicol; wt p53, wild-type p53
Hsp90 is an abundant molecular chaperone important for protecting cells from stress, such as high temperature. Additionally, Hsp90 regulates many signalling pathways. Hsp90 is found in a complex with several oncoproteins, including v-Src, c-Erb2, Raf-1, Akt, Bcr-Abl and tumor suppressor p53 (18-22). The Hsp90 inhibitor - geldanamycin - and its more active derivative, 17-allylamino geldanamycin (17-AAG) are currently in phase I of clinical trials as potential anti-tumor drugs. Hsp90 inhibitors usually induce ubiquitination and degradation of Hsp90 client proteins (23). In tumor cells, Hsp90 exists in a functionally distinct conformational form that is much more efficiently recognized by 17-AAG. This form of Hsp90, which possesses elevated ATPase activity, is found in a multichaperone complex with cochaperones: p23, Hop and probably others (24).

It has been known for years that genotypically mutant p53 coimmunoprecipitates with members of the Hsp70 and Hsp90 families (19). Such interactions lead to the formation of a p53 multichaperone complex that is responsible for the stabilization and sequestration of p53 in the cytoplasm (25-27). Binding of molecular chaperones to mutant p53 inhibits the ability of MDM2 to promote p53 ubiquitination and degradation, resulting in the stabilization of both p53 and MDM2 (28,29). It also has been shown that Hsp90 directly associates with the MDM2 protein (30). Hsp90 inhibitors can partially disrupt these interactions, which results in the degradation of mutant p53 (31,32). With the use of highly purified proteins, we have identified intermediate reactions that lead to the assembly of molecular chaperone complex with p53 protein possessing wild-type or mutant sequence. The presence of Hsp90 in a complex with wild-type p53 inhibits binding of Hsp40 and Hsc70 to p53. However, the conformational mutant of p53, which possesses low affinity towards Hsp90, can form a stable multichaperone complex in which Hsp90 is bound to mutant p53 indirectly (mut p53-Hsp40-Hsc70-Hop-Hsp90). Several independent methods, such as surface plasmon resonance, immunoprecipitation, ELISA and
crosslinking were used to demonstrate that Hsp90 directly, in the absence of any other co-chaperones, can associate with genotypically wild-type p53 but not with mutant p53 protein (33) and unpublished results). The accompanying paper by Muller et al. (34) supports our findings. Moreover, it has been shown by NMR that Hsp90 associates with a truncated version of wt p53. It was suggested that the p53 core domain bound to Hsp90 is predominantly unfolded and lacking helical or sheet secondary structure (35).

Wild-type p53 is a structurally unstable protein, which undergoes conformational changes at elevated temperatures (36,37). We have proposed that during heat shock, cytoplasmic p53 possessing the wild-type sequence could temporarily adopt a mutant conformation, subsequently initiating the formation of a multichaperone complex that could partially stabilize wild-type p53 (19). Results from a recently published paper by Wang and Chen (38) support our hypothesis. They found that heat shock inhibited p53 ubiquitination and initiated the accumulation of p53 at the post-translational level. Two factors influence these events during heat stress: 1) ATM-dependent phosphorylation of p53; 2) formation of the chaperone complex with genotypically wild-type p53, which adopts a conformation characteristic to that of a mutant protein (38).

The evidence for Hsp90 binding to mutant p53 is conclusive, whereas the exact nature of cellular interactions between Hsp90 and genotypically wild-type p53 possessing either wild-type or mutant conformation still remains to be elucidated.

In this study, we demonstrate that the chaperone activity of Hsp90 is required for wild-type p53-dependent transcriptional activity. Specific Hsp90 inhibitors, geldanamycin and radicicol inhibit p53 activity as the transcription factor by dissociation of p53 from its target DNA - promoter sequence sites. Results from the reconstituted in vitro system clearly show that Hsp90 positively regulates p53 DNA binding to a specific promoter sequence after incubation at
physiological temperature of 37°C. Moreover, this Hsp90 activity is ATP dependent and inhibited by geldanamycin.
Materials and Methods

Cell culture experiments

K15, a line of human fibroblasts immortalized by stable expression of hTERT, was a kind gift from prof. H. Kampinga. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂, in Ham’s F10 medium with 15% FBS. For inhibition of topoisomerase I and activation of p53, 2 μM camptothecin (Sigma) was used. For inhibition of Hsp90, 3 μM geldanamycin (Sigma) or 3 μM radicicol (Sigma) were used.

For western blot, the cells were seeded at 50% confluence in Costar 6-well plates. At the indicated times, the cells were lysed directly on the plate in Laemmli sample buffer, and proteins resolved by SDS-PAGE. Immunoblotting was performed using the following antibodies: p53, DO-1 (Santa Cruz Biotechnology), 1:6000; phospho-p53 (Ser-15), #9284 (Cell Signalling Technology), 1:1000; p21, sc-397 (Santa Cruz Biotechnology), 1:100; tubulin: Tub 2.1 (Sigma), 1:2000. Densitometry was performed using the Quantity One software (Biorad).

Real-time RT-PCR,

K15 cells were seeded in 60 mm plates and total RNA was isolated using the Macherey-Nagel NucleoSpin kit. RNA was examined by agarose gel electrophoresis to confirm equal amounts in all samples and lack of degradation. First strand cDNA synthesis was done using the Fermentas RevertAid kit with oligo-dT primers. Real-Time PCR was performed using LightCycler (Roche), as follows: p53 - RT-p53-U, ACCTACCAGGGCAGCTACGG, RT-p53-L, GCTGCACAGGGCAGGTCTTG, annealing temp. 55°C, 1 mM MgCl₂; p21 - RT-p21-U, GGACCTGTCACTGTCTTGTA, RT-p21-L, GGCTTCCTCTTGGAGAAGAT, 53°C, 1 mM MgCl₂; GAPDH - RT-GAPDH-U, GAAGGTGAAGGTCGGAGTCA, RT-GAPDH-L, GAAGATGGTGATGGGATTTC, 51 °C, 2 mM MgCl₂. Detection was done using SYBR-Green.
Chromatin immunoprecipitation (ChIP)

ChIP assay was done as described in (39) with minor modifications. Briefly, K15 cells were cultured in 100 mm plates. After the experimental treatment, the cells were crosslinked with 1% formaldehyde and crosslinking was stopped by addition of glycine. The cells were lysed in RIPA buffer, and DNA was disrupted into pieces of 500-600 bp by sonication. The lysate was cleared by centrifugation and protein A-sepharose beads were added to the lysate together with 1 μl anti-p53 antibody (DO-1, Santa Cruz Biotechnology). After overnight incubation at 4°C, the beads were washed, crosslinking was reversed, and DNA was purified on silica gel columns (A&A Biotechnology). Real-time PCR was performed to detect the p21 promoter fragment using the following primers: p21-ChIP-U, GTGGCTCTGATTGGCTTTCTG, p21-ChIP-L, CTGAAAAACAGGCAGCCCAAG, annealed at 55°C with 1 mM MgCl₂.

Protein purification

Human Hsp90 alpha fused with MBP (plasmid pMALc2x-aHsp90 - a kind gift from P. Csermely) was overexpressed in E.coli BL 21 RIL DE3 strain at 37°C for 3 hours after induction with 0.1 mM IPTG. Cells were harvested by centrifugation at 10 000 g for 10 min and frozen in liquid nitrogen. Bacteria pellet was lysed in buffer A (40 mM Tris pH=7.5, 0.1% β-mercaptoethanol, 5% glycerol, 0.5 mM PMSF) containing 1 mg/ml lysozyme for 1 hour at 4°C with constant stirring, then the centrifugation proceeded for 1 hour at 100 000g.

Supernatant was loaded onto a Q sepharose column equilibrated with buffer A and bound proteins eluted with linear gradient of 0-0.5 M KCl in buffer A. Fractions containing Hsp90 were salted out with 30% (NH₄)₂SO₄ following centrifugation at 70 000g for 20 min. The supernatant was loaded onto a butyl sepharose column which had been equilibrated with buffer A containing 30% (NH₄)₂SO₄ and bound proteins were eluted with linear gradient from 30% to
0% (NH₄)₂SO₄. Hsp90 alpha MBP was applied onto amylose resin (NEB) and eluted with 10 mM maltose. MBP tag was cleaved with factor Xa protease (NEB) according to manufacturers suggestions.

Hsp90 alpha protein of more than 95% purity was concentrated on a Resource Q FPLC column and finally dialysed against buffer B: 25 mM Hepes pH=7.5, 10 % glycerol, 150 mM KCl, 1 mM DTT.

Bovine brain Hsp90 was purified as described (40). Human recombinant Hsc70 (HSPA8) and Hdj1 were overexpressed and purified as described (33). Human recombinant Hsp70 (HSPA1A) was purified exactly as Hsc70, after overexpression in BL21 RIL E.coli strain from pET11b-Hsp70 construct, a kind gift from prof. R. Morimoto. pMALc2x-hdj2 and pET30a-hdj3 - constructs encoding human Hdj2 and Hdj3 were kind gift of prof. K. Terada. Both proteins were purified from E.coli as previously described (41,42). All of these chaperones were tested positively for activity by the luciferase refolding assay (see further).

p53 human recombinant protein was purified essentially as described (43).

**ATPase assay**

ATPase activity was measured as previously described (44). 10 μM Hsp90 was incubated in 20 μl buffer: 40 mM Hepes pH=7.5, 150 mM KCl, 5 mM MgCl₂, 10mM ATP, 0.5 μCi [³²P]γATP/100 μl reaction buffer. Geldanamycin at a concentration of 500 μM was added where indicated and the reaction was carried out at 37°C. At time points 0-120 min, 1 μl samples were spotted on PEI- cellulose plates. Plates were resolved in 1M LiCl: 1M HCOOH, 1:1, dried and spots corresponding to non-hydrolysed ATP and free phosphate were cut out and radioactivity was measured in a liquid scintillation counter (Packard Bioscience). All results were corrected to the spontaneous ATP hydrolysis.
Luciferase refolding assay

Luciferase refolding assay was a modified version of the one described (45). 10 µM Hsp90 was incubated for 30 min at room temperature in the buffer: 25 mM Tris pH=7.8, 8 mM MgSO₄, 1% BSA, 10% glycerol, 0.25 % Triton X-100, containing 2 mM ATP or geldanamycin (concentration ranging from 0.0 to 100 µM). Luciferase (Promega, 12.66 mg/ml) was diluted 400 times in this mixture and incubated for 5 min at 50°C. After cooling down the denatured luciferase mix was diluted 6-fold with a renaturation buffer: 10 mM Tris pH=7.5, 3 mM MgCl₂, 50 mM KCl, 2 mM DTT, 8 mM CP, 0.02 u/µl CK, 2 mM ATP, 4 µM Hsc 70, 2.0 µM Hdj 1. Renaturation was carried out at room temperature. At time points 0- 120 min, 5 µl aliquots were taken out and activity of renatured luciferase was measured in a luminometer (BMG Labtechnologies) after addition of the Bright-Glo substrate (Promega).

p53 DNA binding assay

The DNA binding activity of p53 was quantified by EMSA (gel-shift) assay. 50 ng of human recombinant p53 was diluted in the final volume of 5 µl of EMSA buffer: 50 mM Tris pH 7.5, 5% glycerol, 50mM KCl, 5mM MgCl₂ and 2mM DTT. Samples were supplemented optionally with up to 5 µg Hsp90 (human recombinant alpha or from bovine brain), other chaperones (see Fig.5C) or BSA and 0-20 mM ATP. Such 5 µl samples were then incubated at 4°C or 37°C for 1h in a thermocycler. The activation step followed that included addition of 15 µl mix containing: 1x EMSA buffer, 0.2 MDPM of ³²P labeled p21 sequence (below), 1 µg of unspecific 44-bp dsDNA (sequence below, usage based on (46)) and 100ng of the antibody pAb421 (Ab-1; Oncogene). 20 µl samples with the specific p21 DNA were afterwards incubated for 5-10 min at room temperature (RT), loaded onto a 4% native polyacrylamide TB gel and
electrophoresed at 15mA for 2h at 4°C. Gels were dried and exposed overnight to the Biomax MS-1 Kodak film (Sigma).

For p53 activated by CKII, the activation mix was made, containing per every 4 µl of volume: 50ng of p53, 7U of CKII (Calbiochem), 0.3 U of creatine kinase, 150mM of phosphocreatine (ATP regeneration system; Roche), 1-5 mM of ATP and 1x EMSA buffer. CKII activation was done for 30 min at 25°C and then each 4 µl of CKII activation reaction was supplemented separately with Hsp90/BSA in 1xEMSA buffer up to 5 µl. Afterwards, 1h incubation step either at 4°C or 37°C was performed, followed by addition of 15µl DNA mix without the antibody. Electrophoresis was performed as described above.

For testing the unspecific p53-DNA binding activity, 0.2 MDPM of unspecific, radiolabeled 44-bp dsDNA (below) was used per 1 sample instead of labeled p21 sequence in the activation step. The remaining part of the experiment was performed as mentioned before for specific DNA but no additional unlabeled DNA, antibody or CKII was used in this case.

Best results with Geldanamycin (GA; Sigma) in the EMSA assay were obtained when prior to the addition of p53, Hsp90 was pre-incubated with 83-500 µM GA in the presence of 1xEMSA buffer for 30 min at RT. After the addition of p53 and ATP in 1xEMSA buffer, the final GA concentrations were 25-150 µM. Since the stock solution of GA contained 100% DMSO, in GA titration experiments all samples were supplemented with the same amount of DMSO as added with GA.

Sequences used in EMSA:

p21 promoter derived sequences:

5'-TGGCCATCAGGAACATGTCCCAACATGTTGAGCTCTGGCA-3',

5'-TGCCAGAGCTCAACATGTTGGGACATGTTCCTGATGGCCA-3';

unspecific 44-bp DNA:
5'-GCTTCGAGATGTCCGAGAGGCGAATGAGGCCTTGGAACTCAAG-3',
5'-CTTGAGTTCCAAGGCGCTTCGCTCTCGGAACATCTCGAAGC-3'.

Sequences were annealed to form double stranded DNA in a thermocycler using the following program: 5 min. 94ºC, 5 min. 50ºC, 4 ºC. Presence of the dsDNA was tested with a 16% polyacrylamide TB gel electrophoresis. Sequences used in the EMSA assay were labeled with the T4 Polynucleotide Kinase (PNK; Fermentas) as described in the producer’s manual. 2-week long series of experiments was performed using DNA radioactivity determined at this point. This allowed same amounts of specific DNA per reaction but caused differences in overall intensity of results (e.g. Fig. 6 A and B), as radioactivity of sequences decreased due to the isotope half-life.

**ELISA**

Investigation of the p53 conformation was carried out using a two-site ELISA. First the wells were coated with wild-type p53-conformation specific pAb1620 monoclonal antibody or DO-1 (both of mouse origin, Oncogene Science) at 50 ng per well in carbonate buffer pH 9.2 at 4ºC for 16 h. The wells were blocked for 1 h at room temperature with 100μl of blocking-wash buffer (25mM HEPES-KOH pH 7.6, 5mM DTT, 150 mM KCl, and 2 mg/ml BSA). This was followed by titration of increasing amounts of human recombinant p53, either kept at 4ºC or incubated at 37ºC for 1 h. The p53 dilutions were done in ELISA reaction buffer (25mM HEPES-KOH pH 7.6, 5mM MgCl2 0.05% Triton X-100, 5mM DTT, 150 mM KCl, 2 mg/ml BSA). Detection of p53 protein was carried out using the FL-393 antibody (rabbit origin, Santa Cruz Biotechnology) for 1 h diluted in blocking-wash buffer at room temperature. This was followed by addition of anti-rabbit IgG-HRP secondary antibodies (Santa Cruz Biotechnology). Analysis of bound antibodies was performed by colorimetric detection with the TMB peroxidase.
EIA substrate kit (BioRad), followed by absorbance measurements with microplate reader (BioRad) at 450nm.
Results

Inhibition of Hsp90 in human fibroblasts decreases the wild-type p53 transcriptional activity.

To investigate the possible interaction of the Hsp90 molecular chaperone with wild-type p53 in vivo, we used K15 human fibroblasts. K15 cells express genotypically wild-type p53, which can be immunoprecipitated with wild-type p53-specific pAb1620 antibody, but not with the mutant p53-specific pAb240 antibody (data not shown). This suggests that in K15 cells, p53 exists in a functional, wild-type conformation. The functionality of p53 in the K15 cells was further demonstrated by its ability to induce p21/Waf1 expression and cell cycle arrest upon camptothecin treatment or gamma irradiation (Fig. 1 and data not shown).

Treatment of K15 cells with 2 μM camptothecin (CPT), a drug which induces DNA damage by inhibition of topoisomerase I, results in a p53 upregulation as shown by western blot analysis (Fig. 1A). When the camptothecin treatment was performed in the presence of specific Hsp90 inhibitors, geldanamycin or radicicol (3 μM), p53 was induced to somewhat lower levels, and the time course of the induction was slower (Fig. 1A and result not shown). Importantly, the relative amount of p53 phosphorylated at Serine 15 was not affected by the Hsp90 inhibitors (Fig. 1A). This suggests that Hsp90 might act on p53 directly, rather than through ATR, DNA-PK or ATM kinases, which are known to phosphorylate p53 at this position (47,48). To exclude the possibility that the lower p53 levels were caused by a decrease of its transcription, we quantified p53 mRNA by real-time RT-PCR. As shown in Figure 1B, treatment of cells with geldanamycin or radicicol did not significantly change the level of p53 transcripts, suggesting that those drugs downregulate p53 at the protein level. By fluorescence microscopy and cellular fractionation we showed that geldanamycin or radicicol do not affect the nuclear localization of
p53 (result not shown). These results indicate that Hsp90 might play a role in the stabilization of the wild-type p53 protein.

To test whether Hsp90 indeed can influence the transcriptional activity of p53, we analyzed the binding of p53 to the p21 promoter by chromatin immunoprecipitation (ChIP). As shown in Fig. 1C, the camptothecin-induced binding of p53 to the p21 promoter was almost completely disrupted by geldanamycin. The ChIP experiment for p21 promoter was also successfully performed using anti-Hsp90 antibodies, suggesting a functional interaction between wt p53 and Hsp90 (A.H. unpublished results). Further, we have shown by real-time RT-PCR that geldanamycin or radicicol strongly inhibit the expression of p21 mRNA following the camptothecin treatment (Fig. 1D). This decrease in the p21 level could also be observed by western-blot (Fig. 1A). Taken together, described results suggest that while to some extent Hsp90 stabilizes genotypically wild-type p53 protein level, a stronger effect is visible on the transcriptional activity of the wild-type p53. These phenomena evidently depend on ATP, since both drugs effectively compete with ATP for binding to Hsp90.

**ATPase and chaperone activities of Hsp90 can be inhibited in vitro.**

To answer the question, how does Hsp90 affect the transcriptional activity of p53, we used a reconstituted in vitro system, with highly purified recombinant proteins, to monitor p53 DNA binding to the p21 promoter sequence. For these tests, we used the human recombinant Hsp90 alpha isoform, and for control experiments, we also purified Hsp90 from bovine brain. Purified recombinant Hsp90 alpha as well as bovine Hsp90 possess the ATPase activity, which is inhibited by geldanamycin (Fig. 2A) and radicicol (result not shown). To test the molecular chaperone activity of purified Hsp90 proteins, we used a modified version of a previously described luciferase refolding assay (45). When Hsp90 alpha or bovine Hsp90 was present
during heat denaturation of luciferase (5 min 50°C), the efficient refolding of luciferase was observed at reduced temperatures in the presence of Hsc70 and Hsp40 (Fig. 2B). This refolding reaction was dependent on the presence of Hsp90 during the denaturation process and was severely inhibited when geldanamycin was preincubated with Hsp90 alpha or bovine prior to heat denaturation of luciferase (Fig. 2B).

**Hsp90 proteins promote specific DNA binding activity of wild–type p53 at 37°C.**

The binding of the genotypically wild-type recombinant p53 protein to a p21 promoter sequence was monitored using the gel-shift (EMSA) assay. As described before (49), we used antibody Ab421 or CKII to activate wild-type p53 binding to the promoter DNA, and DO-1 antibody to perform p53 super-shifts as specificity controls (not shown). p53 possesses two DNA binding sites, one sequence-specific, located in the central, core domain of p53 and another non sequence-specific, located at the C-terminal domain of p53 protein. To eliminate the effect of this second non-specific p53 binding site, all p53 DNA binding assays were performed in the presence of a short DNA competitor. In many previous in vitro p53 DNA-binding studies long (>100bp) DNA competitors have been used, such as poly dIdC (50), pBluescript vector (49) or salmon sperm DNA (51). We also initially performed DNA binding experiments with poly dIdC and pBluescript. However, as shown by Anderson and co-workers (46), long competitor DNA molecules inhibited the entry of p53-DNA complexes into the EMSA gel, which caused a decreased quality and reproducibility of our results. This problem was solved by the introduction of short, 44bp competitor DNA fragments to the DNA binding reaction.

It has been previously shown that p53 protein can exist in a constant state of equilibrium between wild-type and mutant conformation (52,53). It is possible that elevated temperatures could shift this equilibrium towards a mutant conformation, hence the amount of p53 possessing
the wild-type conformation should be decreased. Indeed, purified human recombinant wild-type p53 protein loses its wild-type conformation upon incubation at 37°C and higher temperatures (Fig. 3 and result not shown). The immunoprecipitation of the wild-type p53 by pAb 1620, specifically recognizing the wild-type p53 conformation, is significantly reduced following incubation of wild-type p53 at 37°C (Fig. 3A). The same effect was also observed using the modified ELISA test. In this case less p53 was detected by the conformation-specific pAb 1620 while comparable amount of the protein was detected by DO-1 at both temperatures (Fig. 3B). Consistent with these experiments, one-hour incubation at 37°C completely abolished the DNA binding activity of the genotypically wild-type p53, as tested by the gel-shift assay (Fig. 4A lane 2). The presence of increasing amounts of Hsp90 during this incubation step at 37°C significantly enhances the binding of p53 to the p21 promoter sequence (Fig. 4A). This regulation of p53 DNA binding by Hsp90 was found to be ATP-dependent, with at least 3mM ATP required for the distinct effect (Fig. 4B). When we used Poly dI-dC, as an unspecific competitor, the ATP-dependence of this reaction was less pronounced (result not shown).

The results of the ATPase and luciferase refolding assays with Hsp90 alpha and bovine Hsp90 indicate that both preparations possess ATP-dependent chaperone activity. Similarly, both Hsp90 alpha and bovine Hsp90 enhanced p53 DNA binding to the promoter sequence (Fig. 5A). We also determined whether GTP could substitute for ATP in that reaction in order to rule out the involvement of a second, recently proposed nucleotide-binding site on Hsp90 (54). GTP was unable to substitute for ATP during Hsp90-dependent binding of p53 to the promoter sequence (Fig 5B). In addition, geldanamycin, which blocks the N-terminal nucleotide-binding site of Hsp90, inhibits the Hsp90-dependent binding of p53 to the p21 promoter DNA (Fig. 6). Other recombinant human chaperones from Hsp70 and Hsp40 families were not able to efficiently substitute Hsp90 in this reaction (Fig. 5C), neither alone (results for Hdj proteins not shown) nor
in different Hsp/c70 – Hdj combinations, known to refold denatured luciferase (41,42, A.H. unpublished results) and suspected to alter the activity of such chaperone machines (55).

Similar to antibody-activated p53, the DNA binding of CKII-activated p53 was also positively regulated by Hsp90 (Fig. 7). In the control experiment (Fig.7A lane 2), we observed that CKII-treated p53 protein appeared to be more resistant to incubation at 37°C as compared to p53 activated by the pAb421 antibody, which is in agreement with earlier suggestions (43). Surprisingly, the non-specific binding of p53 to dsDNA, for which the unstructured C-terminal part of the protein is responsible (56), is also inhibited after incubation at 37°C (Fig. 7B). However, unlike sequence-specific DNA binding, Hsp90 in presence of 5 mM ATP did not positively regulate the non-specific DNA binding activity of the wild-type p53 (Fig. 7B). These results suggest that the observed Hsp90-mediated enhancement of p53 DNA binding at 37°C is restricted to the p53 core domain that is responsible for binding to specific, promoter-derived DNA molecules. These results fit the domain-specificity of Hsp90-p53 interaction, described in the accompanying paper by Muller et al. (34).

The mode of p53-Hsp90 interaction suggests dynamic mechanism for positive regulation of wild-type p53 by Hsp90.

The ability of Hsp90 to restore p53 binding to the promoter sequence at 37°C can not be explained by a simple, passive protection of the wild-type conformation of p53 at elevated temperatures, analogous to that described for RNA polymerase protection by DnaK (57,58). In the mentioned case the presence of ATP diminishes to a great extent the DnaK-dependent protection of RNA-polymerase at elevated temperatures. However, as shown in Figure 8, the same concentration of ATP that is required for Hsp90-mediated binding of p53 to the promoter sequence (Fig. 4B) also shifts the equilibrium of p53-Hsp90 complex formation towards its
dissociation. The weaker effect of AMP-PNP, the ATP analog, indicates that at least nucleotide hydrolysis and probably its exchange is important for this reaction (Fig. 8). Consequently, 5mM AMP-PNP could not substitute for ATP in the Hsp90-dependent enhanced DNA-binding of p53 in the EMSA assay (result not shown). Hsp90 also did not cause p53 super-shifts in the EMSA assay when Hsp90 was present in reactions (Figs. 4-7) what may suggest a transient nature of the protein-protein interaction. Taken together, these results indicate that the dynamic, repeated binding and dissociation of the Hsp90 to p53 is responsible for the positive regulation of p53 DNA binding activity at physiological temperature of 37°C.
Discussion

The significance of cellular interactions of p53 with heat shock proteins in normal and tumor cells remains unclear. It has been demonstrated that mutant p53 associates to and is stabilized by a multichaperone complex (31,33). p53 protein, encoded by the non-mutated gene, has also been found associated to Hsp90 and presumably other chaperones at elevated temperatures, probably due to the conformational transition of p53 from wild-type to a form characteristic to the mutant protein (38). However, it was not possible to co-immunoprecipitate wild-type conformation p53 with molecular chaperones from cell lysates (26,29,59). A recently published study suggests that Hsp90 inhibitors influence the degradation of p53 temperature sensitive mutant at permissive temperatures in which p53 presumably adopts a wild-type conformation (60). On the contrary, papers by other groups show no significant effect of Hsp90 inhibitors on genotypically wild-type p53 level at physiological temperature (26,38,59), although these inhibitors may cause conformation change of wt p53 (30).

In this paper we show the effect of geldanamycin and radicicol on the activity of genotypically wild-type p53 in human fibroblasts. While the presence of geldanamycin or radicicol had minor effects on the cellular wt p53 level and its phosphorylation at Ser15, these Hsp90 inhibitors dramatically influenced p53 activity as the transcription factor, measured by chromatin immunoprecipitation as well as quantitative analysis of p21 mRNA and protein levels. The observed effect of Hsp90 inhibitors on the p53 transcriptional activity is not due to the inhibition of the nuclear transport of p53 (result not shown). These in vivo data suggest that Hsp90 may play a role in the regulation of p53 promoted transcription. To examine the possible effects of Hsp90 on p53 DNA binding activity, in vitro DNA binding studies were performed with purified human Hsp90 alpha recombinant protein and Hsp90 purified from bovine brain (a
mixture of bovine alpha and beta Hsp90 isoforms). Both Hsp90 protein preparations displayed similar ATPase and luciferase refolding activities that were inhibited by geldanamycin.

The gel-shift assay was used to monitor *in vitro* p53 binding to the p21 promoter DNA sequence. A wild-type p53 conformation, recognized by pAb 1620, is essential for this activity. Incubation of the p53 protein encoded by the non-mutated sequence, at 37°C decreases the amount of p53 protein found in an immuno-complex with pAb 1620 as well as the p53 DNA binding. In fact, incubation of wt p53 for 1h at 37°C completely abolished p53 binding to the p21 promoter-derived sequence. Interestingly, the presence of increasing amounts of Hsp90 during the incubation of p53 at 37°C can positively regulate p53 DNA binding to the promoter sequence, whereas other human recombinant chaperone proteins from Hsp70 and Hsp40 families were not able to substitute for Hsp90 activity. This activity is ATP-dependent and can be inhibited by geldanamycin. These effects correlate with our *in vivo* results, where geldanamycin inhibited p53 binding to the chromatin as well as transcription from the p21 promoter. In order to examine the possibility that transient Hsp90 interactions are required for positive regulation of p53 DNA binding to the promoter sequence at 37°C, we monitored the direct binding of Hsp90 to p53 in the presence or absence of ATP. Similar to Hsp70-substrate complex formation (61,62), the presence of ATP shifted the binding/dissociation equilibrium towards dissociation. These results suggest that the influence of Hsp90 on p53 DNA binding cannot be explained by the passive protection of wild-type p53 conformation, caused by static association with Hsp90. We showed that the presence of ATP, which induces dissociation of Hsp90 from p53, also promotes the ability of p53 to bind to the DNA promoter sequence at 37°C.

The concentrations of geldanamycin and radicicol which are sufficient to inhibit Hsp90–dependent *in vivo* transcriptional activity of p53 are lower than the concentration of Hsp90 inhibitors used in our *in vitro* assays, suggesting that we are only reconstituting the minimum
Hsp90 chaperone systems in vitro. We are in the process of testing the hypothesis that the presence of Hsp90 co-chaperones could influence the inhibitors’ affinity to Hsp90 and Hsp90-dependent p53 binding to the promoter sequence. However, our results already indicate that the influence of Hsp90 on wild-type p53 activity should be taken into consideration while using Hsp90 inhibitors in the therapeutic treatment of cancer, especially if cancer cells possess wt p53.

There are at least two possibilities of explaining the mechanism for Hsp90’s positive regulation of p53 DNA binding to the promoter sequence at 37°C. First, Hsp90 inhibits p53 aggregation or catalyses the disaggregation of p53 protein at elevated temperatures. Such a mechanism was previously discovered for chaperones belonging to prokaryotic (57) and eukaryotic (63) Hsp70 family members; and now is shown for Hsp90 in presence of p53 by Muller et al. (34). Second, the Hsp90 association with wild-type p53 could induce the partial unfolding of p53. Following the dissociation of this Hsp90-p53 complex in the presence of ATP, p53 could spontaneously refold back into a wild-type conformation with a high affinity for the p21 promoter sequence. A similar mechanism of molecular chaperone action was proposed in the case of Hsp100 involved in protein folding and proteolysis (64). The unfoldase activity of Hsp100 molecular chaperone was eventually demonstrated by a subsequent study (65). Recent data from the Ted Hupp laboratory suggest that Hsp90 in the presence of MDM2 could indeed partially unfold p53 (30). We propose that partial unfolding of p53 could be catalyzed by Hsp90, and the subsequent spontaneous refolding of p53 back into a wild-type-like conformation may prevent p53 aggregation thus increasing p53 DNA binding to the promoter sequence (see Fig. 9). More importantly, these chaperone-mediated actions would decrease the probability for the formation of kinetically trapped, mutant-like intermediates and that would allow a shift in the conformational equilibrium towards the active, wild-type p53 conformation. These events would
ultimately promote the p53 transcriptional activity and allow for the ubiquitination and degradation of p53 protein. In addition, the retention of p53 in a wild-type conformation by transient Hsp90 interaction would also inhibit the formation of a multichaperone-p53 complex, which prevents p53 degradation and import to the nucleus (Fig. 9).
References


Acknowledgements

We thank Johannes Buchner for helpful discussions and sharing of unpublished results concerning interactions of Hsp90 with p53. We thank Peter Csermely for the plasmid pMALc2x-aHsp90 encoding human Hsp90 alpha, Ted Hupp for plasmid constructs for p53 overexpression, Harm Kampinga for the kind gift of the K15 cell line, Richard Morimoto for pET11b plasmid encoding human Hsp70 and Kazutoyo Terada for constructs for Hdj2 and Hdj3 overexpression. This work was supported by KBN grant No 3P04B02122 for AZ, Foundation for Polish Science grant for MZ, V Framework grant QLRT-2001-02833 and grants of the DFG for Johannes Buchner.

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Figure legends

Fig. 1 Hsp90 inhibitors decrease p53 activity as the transcription factor. K15 cells were cultured in the presence or absence of 2 μM camptothecin (CPT), 3 μM geldanamycin (GA) or 3 μM radicicol (R) for the indicated time periods. (A) The time course of total p53 (p53), p53 phosphorylated at Ser15 (Ser15-P-p53) and p21/Waf1 protein (p21) induction was analyzed by western blotting. Tubulin was detected as the total protein level control; (B) Cellular p53 mRNA levels 3.5 hours after the addition of camptothecin were quantified by real-time RT-PCR. The results were normalized to the GAPDH mRNA. (C) p53 binding to the p21 promoter sequence 4 hours after camptothecin treatment was detected by chromatin immunoprecipitation (ChIP) and real-time PCR. (D) Cellular p21/Waf1 mRNA levels 3.5 hours after the addition of camptothecin were quantified by real-time RT-PCR. All results are representative of at least three independent experiments.

Fig. 2 ATPase and luciferase refolding activities of Hsp 90 alpha (α) and Hsp90 purified from bovine brain (bov) are inhibited by geldanamycin.

(A) Hsp90 proteins at concentration 10 μM were incubated in reaction buffer with 10mM ATP and 0.1 μCi [32P]γATP at 37 °C and 0.5 mM geldanamycin was added when indicated. At indicated time points 1/20 of the reaction mixture was spotted on PEI cellulose plates. After resolving and drying the plates were cut and spots corresponding to free phosphate and non-hydrolysed ATP were counted for radioactivity.

(B) Hsp 90 preparations were incubated with 2 mM ATP or 100 μM geldanamycin. Luciferase (Promega 12.66 mg/ml) was 400 times diluted with this mixture and incubated for 5 min at 50° C. After cooling down, the denatured luciferase was 6 times diluted with the renaturation buffer containing Hsc70, Hdj 1, ATP and ATP regeneration system in
concentrations described in Materials and Methods. The refolding reaction was carried out at 25°C. At indicated time points 1/6 of the reaction was tested for luciferase activity using luciferase substrate. Not shown controls contained Hsp90s alone, with no other chaperones and had refolding activity similar to “no Hsp90” reactions.

Fig. 3 Wild-type human recombinant p53 loses its wild-type conformation upon incubation at 37°C.

(A) p53 was incubated for indicated periods of time at 4°C or 37°C and subsequently immunoprecipitated with the wt-specific antibody pAb 1620. Detection after SDS-PAGE and blotting of the IPs was performed with antibody DO-1 – detecting total amount of the immunoprecipitated p53. Less wild-type conformation p53 was immunoprecipitated when p53 was kept at 37°C for a longer time.

(B) ELISA wells were coated with anti-p53 antibodies pAb 1620 (50 ng, wt specific) or DO-1 (50 ng, pan-specific) and increasing amounts of p53 were added onto the wells. Upon incubation at 37°C for one hour the amount of mutant conformation p53 rises (decrease in pAb 1620 antibody reactivity), which corresponds to the lower curve on the graph (open circles) in comparison with p53 kept at 4°C (filled circles). This effect was not due to decreased antibody accessibility to p53 caused by aggregation, since DO-1 antibody binding did not significantly change after 37°C incubation (open and filled squares). All presented values are means of 4 repeats. The OD is normalized to the maximum values for both antibodies appropriately.
Fig. 4 Human recombinant Hsp90 alpha restores the specific DNA binding activity of the human recombinant p53 after the 1h inactivation at 37°C in vitro.

(A) p53 was incubated for 1h at 4°C (lane 1) or 37°C without or in the presence of the increasing amount of Hsp90 alpha (H90α, lanes 3-6). All reactions contained 5mM ATP. The p53/Hsp90 mass ratio is indicated for lanes with the increasing amount of Hsp90. At 1/50 protein mass ratio 0.05 µg of p53 was used (0.22 µM of the monomer) and 2.5 µg Hsp90 alpha (5.6 µM of the monomer).

(B) p53 was incubated for 1h at 4°C or 37°C, with or without Hsp90 alpha, as indicated. The effect of different amounts of ATP has been tested, as shown by bars and by mM amounts of ATP used. 5 µg of BSA was added instead of 2.5 µg Hsp90 in lane 7. At 3-5 mM ATP the Hsp90-dependent p53 rescue increases dramatically (lanes 9-11).

The experiments were performed as described in Materials and methods.

All shown EMSA bands in this and following Figures (unless indicated otherwise) correspond to the size of p53 tetramer bound to the DNA, super-shifted by the activating antibody Ab421.

Fig. 5 Bovine brain Hsp90 has the same effect in vitro on p53 as Hsp90 alpha. GTP does not replace ATP in this reaction and other human chaperones can not substitute Hsp90.

(A) In lanes 5-6 bovine brain purified Hsp90 was used instead of Hsp90 alpha. 2.5 µg Hsp90s and 0-5 mM ATP was used. Remaining reaction conditions like in Fig.4.

(B) Lanes 1-3 contained 5mM GTP that does not substitute for 5mM ATP (lane 4) in the Hsp90 alpha dependent recovery of p53. Nevertheless, it is visible that GTP has some protective activity by itself, so p53 does not get as much inactivated as in the presence of ATP or no nucleotide (see A).
(C) Reactions carried out like in A - 1h at indicated temperature, 5mM ATP in all lanes. Bovine brain Hsp90 (lane 3) was used at 2.5 μg (5.6 μM). Human recombinant Hsp70 (HSPA1A) and Hsc70 (HSPA8) were used at 6 μM in lanes 4-5 as substitutes for Hsp90. Combinations of chaperones from Hsp70 and Hsp40 family (Hdj1, 2 and 3) known to efficiently refold denatured luciferase were used in lanes 6-11, at molar ratio Hsp/c70:Hsp40 2:1 (6 μM:3 μM). Due to Hdj1 high pI and its unspecific DNA binding ability, this protein was used in lane 12 without p53.

Fig. 6 The in vitro p53 rescuing activity of Hsp90 alpha and bovine brain Hsp90 can be inhibited by increasing amount of geldanamycin (GA).

(A) 1.25 μg of Human Hsp90 alpha (α) was preincubated for 30 min. with the indicated amount of GA (Materials and methods) and used for p53 rescue from 37°C inactivation in the presence of 5mM ATP (lanes 3-8). In all other lanes reactions were carried out identically, with 5mM ATP, but excluding Hsp90. Lane 9 is a control confirming lack of the negative effect of GA on p53 binding to the DNA in the absence of Hsp90.

(B) Reactions like in A but bovine brain Hsp90 is used instead of Hsp90 alpha. In case of this isoform less GA was required to fully inhibit Hsp90.

For the reason of differences in overall intensity of bands between A and B see Materials and Methods.

Fig. 7 Only the specific p53 DNA binding, activated by both Ab421 and CKII, is rescued by Hsp90 in vitro.

(A) The specific binding of p53 to the labeled p21 promoter DNA was activated by the antibody Ab421 (lanes 4-6; super-shifted by the antibody) and CKII-dependent phosphorylation
(lanes 1-3; not super-shifted) and both could be inactivated by 1h incubation at 37°C (lanes 2 and 5). Although difference between binding strength at 4°C and 37°C is smaller in case of CKII than in Ab421 activation (phosphorylation may protect against heat inactivation, see text for reference), Hsp90 alpha rescues p53 specific binding in both cases (lanes 3 and 6). 5mM ATP was present in all reactions and in case of CKII activation an ATP-regeneration system was used to maintain the ATP concentration.

(B) The ³²P labeled unspecific DNA sequence and no unspecific competitor was used instead of the specific p21 promoter derived DNA (see Materials and methods). p53 binding to the unspecific DNA was also inactivated by 37°C (2), but it can not be restored by Hsp90 alpha (3). All reactions contained 5mM ATP.

Protein amounts and ratios for A and B like in Fig.5A.

**Fig.8 ATP dissociates the Hsp90-p53 complex.**

Hsp90 (500 ng) was coated onto an ELISA plate in 50ml buffer (25 mM HEPES-KOH pH 7.5 and 100 mM KCl). The incubation proceeded for 2h at room temp. After the washing and blocking procedure (25 mM HEPES-KOH pH 7.5, 100 mM KCl and 2mg/ml BSA), the indicated amounts of wild-type p53 in the reaction buffer (25 mM HEPES-KOH pH 7.5, 150 mM KCl, 10mM MgCl₂, 5% glycerol, 0.05% Triton X-100, 1 mg/ml of BSA) were added in the presence of 5 mM ATP or its analog AMP-PNP for 1h at 25°C. The detection of p53 bound to Hsp90 followed as described (33). All shown values are means of 4 repeats.

**Fig. 9 The proposed model for a role of molecular chaperones in maintaining of p53 in cells.**
The wild-type structure of p53 is represented by circles while the mutant conformation by squares. p53 sequence, oncoproteins and other factors may shift the equilibrium between wild-type, mutant conformation and aggregation. Among those factors are molecular chaperones. Immediate reactions involving Hsp90 in positive regulation of wt p53 are not yet known, hence the question mark. More details included in the discussion.
Fig. 1

A. Western blot analysis showing Ser15-P-p53, p53, p21, and tubulin levels after treatment with CPT, CPT + GA, CPT + R, or no CPT for different time periods (CPT h: 0, 1, 2, 4, 6, 8).

B. Bar graph depicting the relative mRNA levels of p53/eGAPDH for CPT, CPT + GA, CPT + R, and no CPT.

C. Bar graph showing the relative p21 promoter DNA ChIP with p53 for CPT, CPT + GA, CPT + R, and no CPT.

D. Bar graph illustrating the relative mRNA levels of p21/eGAPDH for CPT, CPT + GA, CPT + R, and no CPT.
Fig. 2

**A**

- Hsp90 α
- Hsp90 bov
- Hsp90 α + GA
- Hsp90 bov + GA
- BSA

**B**

- Hsp90 α
- Hsp90 bov
- Hsp90 α + GA
- Hsp90 bov + GA

**Legend**

- V (mmol P/mmol Hsp90)
- time (min)

- Luciferase efolded (%)
Fig. 3

A

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B

- 1620(4°C)
- 1620(37°C)
- DO-1(4°C)
- DO-1(37°C)

p53-antibody complex (OD 450 nm) vs ng p53
### Fig. 5

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Fig. 6

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### Fig. 7

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#### A

1. 2. 3. 4. 5. 6.

#### B

1. 2. 3.
Fig. 8

The graph shows the formation of the Hsp90-p53 complex (OD 450 nm) as a function of p53 (ng) concentration. The graph compares the complex formation in the presence of different nucleotides: no nucleotide, AMP-PNP, and ATP. The y-axis represents the OD 450 nm, and the x-axis represents the p53 concentration (ng).

Key:
- **no nucleotide**
- **AMP-PNP**
- **ATP**
Fig. 9

A mutant p53 multichaperone complex

- Hsp90
- Hop
- Hsc70
- Hsp40

no degradation

p53 aggregates

- Hsp90
- Hsp40
- Hsc70
- Hop
- Hsp90?

transcription activation

 promoter DNA

- MDM2
- Chip?
- Bag-1?
- COP1?

degradation

transcription inhibition

- wt
- mut
Hsp90 chaperones wild-type p53 tumor suppressor protein
Dawid Walerych, Grzegorz Kudla, Małgorzata Gutkowska, Bartosz Wawrzynow, Lin Muller, Frank W. King, Aleksandra Helwak, Joanna Boros, Alicja Zylicz and Maciej Zylicz

J. Biol. Chem. published online September 9, 2004

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