Hsp90 Chaperones Wild-type p53 Tumor Suppressor Protein*

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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 lead 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 α 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 Hsp90α activity. We show that the incubation of recombinant p53 at 37 °C 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 37 °C. 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 article state that chaperone activity of Hsp90 is important for the transcriptional activity of genotypically wild-type p53.

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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 the 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-p531 in the cytoplasm, because of 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 post-translational level by MDM2 (12, 15–17).

Hsp90 is an abundant molecular chaperone important for protecting cells from stress, such as high temperature. Additionally, Hsp90 regulates many signaling 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 17-allylamino-geldanamycin (17-AAG) is currently in phase II of clinical trials as a potential anti-tumor drug. 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 co-immunoprecipitates 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

1 The abbreviations used are: wt-p53, wild-type p53; Hsp90, heat shock protein 90; AMP-PNP, adenylyl-imidodiphosphate; ChIP, chromatin immunoprecipitation; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; GA, geldanamycin; mut p53, p53 in mutant conformation; R, radicicol; DTT, dithiothreitol; BSA, bovine serum albumin.
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 wt-p53 inhibits binding of Hsp40 and Hsc70 to p53. However, the conformational mutant of p53, which possesses low affinity toward 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 cross-linking were used to demonstrate that Hsp90 directly, in the absence of any other co-chaperones, can associate with genotypically wt-p53 but not with mutant p53 protein (33). The accompanying article 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 wt-p53 (19). Results from a recently published article 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 and 2) formation of the chaperone complex with genotypically wt-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 wt-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 wt-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% fetal bovine serum. For inhibition of topoisomerase I and activation of p53, 2 μM camptothecin (Sigma) were used. For inhibition of Hsp90, 0.3 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), 9294 (Cell Signaling 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 (Bio-Rad).

Real-time RT-PCR—K5 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 Diagnostics). As follows: p53, RT-p53-U, ACCTACCGGGGCACGTCG, RT-p53-L, GCTGACAGGGGACGTCTTGG, annealing temperature 55 °C, 1 mM MgCl₂; p21, RT-p21-U, GGACCTGCTACGTGGTGTTGTGTA, RT-p21-L, GGCCTCCTCTTGGAGAAGAT, 53 °C, 1 mM MgCl₂; GAPDH, RT-GAPDH-U, GAGAAGTGAAGGTCGGAGTCA, RT-GAPDH-L, GAAGATGTTAGGACGTTTC, 51 °C, 2 mM MgCl₂. Detection was performed using SYBR-Green.

Chromatin Immunoprecipitation (ChIP)—ChIP assay was done as described in Ref. 39 with minor modifications. Briefly, K5 cells were cultured in 100-mm plates. After the experimental treatment, the cells were cross-linked with 1% formaldehyde and cross-linking was stopped by addition of glycine. The cells were lysed in radioimmune precipitation assay 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 of anti-p53 antibody (DO-1, Santa Cruz Biotechnology). After overnight incubation at 4 °C, the beads were washed, cross-linking 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, GTGGCTCTTATGGCCTCTTGTG, p21-ChIP-L, CTGAAAACAGGCAGCCCAAG, annealed at 55 °C with 1 mM MgCl₂.

Protein Purification—Human Hsp90α was fused with MBP (plasmid pMALc2x-hs90α; a kind gift from P. Csermely) overexpressed in Escherichia coli BL 21 RIL DE3 strain at 37 °C for 3 h after induction with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside. 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 phenylmethylsulfonyl fluoride) containing 1 mg/ml lysozyme for 1 h at 4 °C with constant stirring, then the centrifugation proceeded for 1 h at 100,000 g. Supernatant was loaded onto a Q-Sepharose column equilibrated with buffer A containing 30% (NH₄)₂SO₄, and bound proteins were eluted with linear gradient from 30 to 0% (NH₄)₂SO₄.

Bovine brain Hsp90α was purified as described (40). Human recombinant Hsc70 (HSPA8), and Hdj1 were overexpressed and purified as described (33). Human recombinant Hsp70 (HSPA1A) was purified essentially as described (43).

ATPase Assay—ATPase activity was measured as previously described (44). 10 μM Hsp90α was incubated in 20 μl of buffer: 40 mM Hepes pH 7.5, 150 mM KCl, 5 mM MgCl₂, 10 mM ATP, 0.5 μCi of [γ-32P]ATP/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. After resolving and drying the plates were cut, and radioactivity was measured in a liquid scintillation counter (Packard Bioscience). All results were corrected to the spontaneous ATP hydrolysis.
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, 50 mM KCl, 5 mM MgCl₂, and 2 mM DTT. Samples were supplemented optionally with up to 5 μg of Hsp90 (human recombinant α 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 or 37 °C for 1 h in a thermocycler. The activation step followed that included addition of 15 μl of mixture containing: 1× EMSA buffer, 0.2 Mdm of p53-labeled p21 sequence (below), 1 μg of nonspecific 44-bp dsDNA (sequence below, usage based on Ref. 46), and 100 ng of the antibody pAb421 (Ab-1; Oncogene). 20-μl samples with the specific p21 DNA were afterward incubated for 5–10 min at room temperature, loaded onto a 4% native polyacrylamide Tris borate gel and electrophoresed at 15 mA for 2 h 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: 50 ng of p53, 7 units of CKII (Calbiochem), 0.3 units of creatine kinase, 150 mM of phosphocreatine (ATP regeneration system; Roche Applied Science), 1–5 μl of ATP and 1× EMSA buffer. CKII activation was done for 30 min at 25 °C, and then each 4 μl of CKII activation reaction were supplemented separately with Hsp90/BSA in 1× EMSA buffer up to 5 μl. Afterward, a 1-h incubation step either at 4 or 37 °C was performed, followed by addition of 15 μl of DNA mixture without the antibody. Electrophoresis was performed as described above.

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

Best results with geldanamycin in the EMSA assay were obtained when prior to the addition of p53, Hsp90 was preincubated with 83–500 μM GA in the presence of 1× EMSA buffer for 30 min at room temperature. After the addition of p53 and ATp in 1× EMSA buffer, the final GA concentrations were 25–150 μM. Since the stock solution of GA contained 100% Me₂SO, in GA titration experiments all samples were supplemented with the same amount of MeSO as added with GA.

Sequences used in EMSA: p21 promoter-derived sequences, 5′-TG-GCCATCACAGAACATGTTCCACATGGAGCTCTGGCA-3′ and 5′-TGCCAGAGCTCAACATGTTGGGACATGGTCTCGAGGCCA-3′; nonspecific 44-bp DNA, 5′-GCTTCGAGATGTTCCGAGAGGCAGAATGG-3′; nonspecific 44-bp DNA, 5′-GCTTCGAGATGTTCCGAGAGGCAGAATGG-3′; nonspecific 44-bp DNA, 5′-GCTTCGAGATGTTCCGAGAGGCAGAATGG-3′; nonspecific 44-bp DNA, 5′-GCTTCGAGATGTTCCGAGAGGCAGAATGG-3′. Sequences were annealed to form double-stranded DNA in a thermocycler using the following program: 5 min 94 °C, 5 min. 50 °C, 4 °C. Presence of the dsDNA was tested with a 16% polyacrylamide Tris borate gel electrophoresis. Sequences used in the EMSA assay were labeled with the T4 polynucleotide kinase (PNK; Fermentas) as described in the producer’s manual.

ELISA—Investigation of the p53 conformation was carried out using a two-site ELISA. First the wells were coated with wt-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 4 °C for 16 h. The wells were blocked for 1 h at room temperature with 100 μl of blocking wash buffer (25 mM Hepes-KOH pH 7.6, 5 mM DTT, 150 mM KCl, and 2 mg/ml BSA). This was followed by titration of increasing amounts of human recombinant p53, either kept at 4 °C or incubated at 37 °C for 1 h. The p53 dilutions were done in ELISA reaction buffer (25 mM Hepes-KOH pH 7.6, 5 mM MgCl₂ 0.05% Triton X-100, 5 mM 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-horseradish peroxidase secondary antibodies (Santa Cruz Biotechnology). Analysis of bound antibodies was performed by colorimetric detection with the TMB peroxidase EIA substrate kit (Bio-Rad), followed by absorbance measurements with a microplate reader (Bio-Rad) at 450 nm.

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 wt-p53 in vivo, we used K15 human fibroblasts. K15 cells express genotypically wt-p53, which can be immunoprecipitated with wt-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 radiation (Fig. 1 and data not shown).

Treatment of K15 cells with 2 mM camptothecin (CPT), a drug which induces DNA damage by inhibition of topoisomerase I, results in a p53 up-regulation 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 results not shown).

To test whether Hsp90 indeed can influence the transcriptional activity of p53, we analyzed the binding of p53 to the p21 promoter by 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 the p21 promoter was also successfully performed using anti-Hsp90 antibodies, suggesting a functional interaction between wt-p53 and Hsp90.2 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 wt-p53 protein level, a stronger effect is visible on the transcriptional activity of the wt-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 α isoform, and for control experiments, we also purified Hsp90 from bovine...
p53-Hsp90 Functional Interactions

| Temp. | ATP (mM): Hsp90 | 4°C | 37°C | 37°C | 37°C | 37°C | 37°C |
|-------|----------------|-----|-----|-----|-----|-----|-----|
|       |                | 5   | 5   | 0   | 5   | 0   | 5   |
|       | Alpha          |     |     |     |     |     |     |
|       | Bov.           |     |     |     |     |     |     |

| Temp. | Nucleotide: Hsp90 (2.5 μg) | 4°C | 37°C | 37°C | 37°C | 37°C |
|-------|-----------------------------|-----|-----|-----|-----|-----|
|       | GTP                         |     |     |     |     |     |
|       | GTP                         |     |     |     |     |     |
|       | GTP                         |     |     |     |     |     |
|       | ATP                         |     |     |     |     |     |

**A**

**B**

**C**

**Fig. 5.** Bovine brain Hsp90 has the same effect in vitro on p53 as Hsp90α. GTP does not replace ATP in this reaction, and other human chaperones cannot substitute Hsp90. A, in lanes 5 and 6 bovine brain-purified Hsp90 was used instead of Hsp90α. 2.5 μg of Hsp90α and 0–5 mM ATP were used. Remaining reaction conditions were similar to Fig. 4. B, lanes 1–3 contained 5 mM GTP that does not substitute for 5 mM ATP (lane 4) in the Hsp90-dependent recovery of p53. Nevertheless, it is apparent that GTP has some protective activity by itself, so p53 is not inactivated as much as in the presence of ATP or no nucleotide (see A). C, reactions carried out as in A; 1 h at indicated temperature, 5 mM 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 μg in lanes 4 and 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 (6:3 M). Human recombinant Hsp70 (HSPA1A) and Hsc70 (HSPA8) were used at 6 μg in lanes 4 and 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 (6:3 M). Lane 12 represents unspecific Hdj1 binding to the p21 promoter DNA in the absence of p53.

We also initially performed DNA binding experiments with poly(dI-dC) and pBluescript. However, as shown by Anderson et al. (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, 44-bp 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 toward a mutant conformation, hence the amount of p53 possessing the wild-type conformation should be decreased. Indeed, purified human recombinant wt-p53 protein loses its wild-type conformation upon incubation at 37 °C and higher temperatures (Fig. 3 and results not shown). The immunoprecipitation of the wt-p53 by pAb 1620, specifically recognizing the wt-p53 conformation, is significantly reduced following incubation of wt-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 whereas comparable amount of the protein was detected by DO-1 at both temperatures (Fig. 3B). Consistent with these experiments, 1-h incubation at 37 °C completely abolished the DNA binding activity of the genotypically wt-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 bind-
The ability of Hsp90 to restore p53 binding to the promoter sequence at 37 °C cannot 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 Fig. 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 toward 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, 5 mM AMP-PNP could not substitute for ATP in the Hsp90-dependent enhanced DNA-binding of p53 in the EMSA assay (results not shown). Hsp90 also did not cause p53 supershifting in the EMSA assay when Hsp90 was present in reactions (Figs. 4–7), which 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 temperatures 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). In contrast, articles by other groups show no significant effect of Hsp90 inhibitors on genotypically wt-p53 level at physiological temperature (26, 38, 59), although these inhibitors may cause conformational change of wt-p53 (30).

In this article we show the effect of geldanamycin and radicicol on the activity of genotypically wt-p53 in human fibroblasts. Whereas the presence of geldanamycin or radicicol had minor effects on the cellular wt-p53 level and its phosphorylation at Ser-15, 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 caused by the inhibition of the nuclear transport of p53 (results not shown). These in vivo data suggest that Hsp90 may play a role in the

**FIG. 6.** The *in vitro* p53 rescuing activity of Hsp90α and bovine brain Hsp90 can be inhibited by increasing amounts of GA. A, 1.25 µg of human Hsp90α was preincubated for 30 min with the indicated amount of GA (see “Materials and Methods”) and used for p53 rescue from 37 °C inactivation in the presence of 5 mM ATP (lanes 3–8). In all other lanes reactions were carried out identically with 5 mM 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 as in A but bovine brain Hsp90 is used instead of Hsp90α. In the case of this isoform less GA was required to fully inhibit Hsp90. For explanation of differences in overall intensity of bands between A and B see “Materials and Methods.”
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 Hsp90α recombinant protein and Hsp90 purified from a bovine brain (a mixture of bovine α and β 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 wt-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 immunocomplex with pAb 1620 as well as the p53 DNA binding. In fact, incubation of wt-p53 for 1 h 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 toward dissociation. These results suggest that the influence of Hsp90 on p53 DNA binding cannot be explained by the passive protection of wt-p53 conformation, caused by static association with Hsp90. 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 toward dissociation. These results suggest that the influence of Hsp90 on p53 DNA binding cannot be explained by the passive protection of wt-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
no degradation
degradation

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 whereas 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 marks. More details are included in “Discussion.”

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