Hsp90 Regulates the Activity of Wild Type p53 under Physiological and Elevated Temperatures*

Received for publication, July 8, 2004, and in revised form, September 8, 2004
Published, JBC Papers in Press, September 9, 2004, DOI 10.1074/jbc.M407687200

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The activity and structural integrity of the tumor suppressor protein p53 is of crucial importance for the prevention of cancer. p53 is a conformational flexible and labile protein, in which structured and unstructured regions function in a synergistic manner. The molecular chaperone Hsp90 is known to bind to mutant and wild type p53 in vitro. Using highly purified proteins we analyzed the interaction and the binding sites between both proteins in detail. Our results demonstrate that Hsp90 binds to a folded, native-like conformation of p53 in vitro with micromolar affinity. Specifically, the DNA-binding domain of p53 and the middle and carboxy-terminal domains of Hsp90 are responsible for this interaction, which is essential to stabilize p53 at physiological temperatures and to prevent it from irreversible thermal inactivation. Our results are in agreement with a model in which Hsp90 is required to maintain the folded, active state of p53 by a reversible interaction, thus introducing an additional level of regulation.

The tumor suppressor protein p53 is a transcription factor involved in cell cycle regulation and the initiation of apoptotic cell death (1–4). Mutated or otherwise, deactivated p53 is observed in over 50% of human cancers (5, 6). It can interact with and inactivate the protein from the wild type allele (7, 8). Wild type p53 is a labile protein with a high turnover rate; it is degraded rapidly via proteolysis and the ubiquitin-proteasome pathway (9). Due to this high turnover only low concentrations of p53 reside usually in the cell. However, mutated p53 is not as strongly affected by proteolysis or ubiquitination and therefore accumulates in the cell (10).

p53 consists of 393 residues that can be divided in four major functional domains (see Fig. 1A) (11–13). As a transcription factor, p53 possesses an amino-terminal region (NTD), residues 1–93) (14), which contains a transcription activation domain and a proline-rich domain. Highly specific DNA binding is mediated by the core domain (DBD, residues 102–292) (15, 16), which recognizes a consensus DNA sequence (17). The high affinity binding of p53 to this sequence requires the tetrameric state of p53 (17). The tetramerization domain (18), which is followed by a flexible regulatory domain (RD), is located within the carboxy-terminal domain (residues 320–393). Stress-mediated modifications in the regulatory domain, e.g. phosphorylation or binding of other proteins, transform p53 from the common inactive form to the activated form, which is able to induce the expression of target proteins or to mediate apoptosis (19).

Interestingly, CD spectroscopy revealed that p53 contains large unstructured regions in its native state (12). Further analysis using NMR spectroscopy in combination with p53 fragments showed that the NTD is natively unfolded (20). Thus, in p53 only the DBD and the tetramerization domain are highly structured (21, 22). This classifies p53 as a member of the growing class of proteins, in which structured and unstructured regions function in a synergistic manner (20). For p53 this allows the interaction with a multitude of regulators (2, 23, 24).

It has been shown that mutated p53 interacts specifically with the molecular chaperone Hsp90 in vivo (25–27). Hsp90 is essential in eukaryotes (28), and it is one of the most abundant proteins in unstressed cells (29, 30). It exhibits high homology between higher and lower eukaryotes (29, 30), and it is one of the most abundant proteins in unstressed cells (29, 30). It exhibits high homology between higher and lower eukaryotes: e.g. yeast Hsp90 (yHsp90) is 60% identical to human Hsp90 (31). Furthermore, the function of Hsp90 as a chaperone for a large number of client proteins, ranging from transcription factors to kinases, seems to be conserved between yeast and humans (32).

The chaperone is an elongated dimer, which can be divided into three major domains (see Fig. 1B) (33–36). The amino-terminal domain of Hsp90 contains the ATP binding pocket (35). This pocket also binds the Hsp90 drugs geldanamycin and radicicol (37, 38), which block ATP binding and therefore inhibit the ATPase activity. Derivatives of these inhibitors show antitumor activity in various preclinical models (39). The ATP-binding domain is connected to the remainder of the protein via a highly charged and protease-sensitive linker domain (Fig. 1B, CR) (40). The function of the adjacent middle domain of Hsp90 is not completely understood. It has been reported that this domain is involved in the ATPase activity of Hsp90 (41, 42). The dimerization site is located in the carboxy-terminal region of the protein (34). Additionally, the carboxy-terminal amino acids provide the binding site for partner proteins containing TPR domains (tetratricopeptide repeat). These TPR domains...
are a common feature of many Hsp90 cochaperones such as Hop (32, 43, 44).

The relevance of the interaction of Hsp90 and p53 is still enigmatic. As mentioned above, Hsp90 binds tightly to mutated p53 (25, 26, 45–47) and inhibition of Hsp90 function accelerates mutant p53 degradation (48). NMR experiments suggest that the completely denatured DBD of p53 binds to Hsp90 (49). Interestingly, Hsp90 also binds to wild type p53 (46). The binding to the wild type protein seems to be more transient compared with mutant proteins. There is also in vivo evidence that heat shock influences binding to Hsp90 (27).

In this study we analyzed the interaction of p53 and Hsp90 in detail. We show that Hsp90 is vital for the stability and the DNA-binding ability of wild type p53 at physiological and heat shock temperatures.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification—**Wild type p53 (residues 1–393), p53-core (residues 94–312), p53-C312 (residues 1–312), N93-p53 (residues 93–339), and p53-C363 (residues 1–363) were expressed and purified as described previously (42). Human Hsp70 was expressed and purified as described elsewhere (12, 20).

The DNA sequences for amino acids 313–393 and 1–363 of human p53 were amplified from plasmid p11435 (12) by polymerase chain reaction and inserted into the multiple cloning site of a pQE30 expression vector driven by the isopropyl β-D-thiogalactopyranoside-inducible T5 phage-promotor (Qiagen). The resulting plasmids, pTC and pDC, were loaded onto a nickel-coated HiTrap Chelating HP column (Amersham Biosciences), respectively. N313-p53 and p53-C363 were expressed as soluble protein in Escherichia coli HB101, transfected with the plasmid pTc or pDC, respectively, and the helper plasmid pUBSS20 (50). Bacteria were grown in Luria broth medium at 37 °C up to an optical density of 0.8 at 600 nm. After lysis, the resulting supematant was centrifuged at 4000 × g for 15 min at 4 °C, and the supernatants were incubated with 1 ml isopropyl β-D-thiogalactopyranoside, cells were harvested by centrifugation, resuspended in 20 mM sodium phosphate, pH 7.5, 300 mM sodium chloride, complete protease inhibitor tablets (Roche Applied Science), and disrupted by high pressure disper- sion. After lysis, the supernatant containing soluble p35-C363 was loaded onto a nickel-coated HiTrap Chelating HP column (Amer- sham Biosciences). Bound p53-C363 was eluted with a linear imidazole gradient.

Protein-containing fractions were pooled and purified to homogeneity by size exclusion chromatography on a High Load 16/60 Superose 6 column containing fractions were pooled and purified to homogeneity by size exclusion chromatography on a High Load 16/60 Superose 6 column (Amersham Biosciences), respectively. N313-p53 and p53-C363 were expressed as soluble protein in Escherichia coli strain BL21-CodonPlus (DE3) and purified as described elsewhere (52).

**Analytical Gel Filtration—**Analytical gel filtration analysis was performed at 20 °C with a flow rate of 0.75 ml/min using a TSK2000SW (TosoHaas, Amsterdam, Netherlands) or a BioSep SEC-3000 (Ph- nomenex, Aschaffenburg, Germany) column attached to a high perform- ance liquid chromatography system (Amersham Biosciences). The column was equilibrated with sample buffer consisting of 20 mM sodium phosphate, pH 7.5, 300 mM sodium chloride, 2 mM EDTA. Purified proteins with a concentration of ~2–5 μM were injected in a total volume of 20 μl. Elution of proteins was monitored by fluorescence emission at 320 nm (excitation, 280 nm).

**FITC Labeling of Hsp90—**FITC (fluorescein-5-isothiocyanate) label- ing of Hsp90 was performed using the FluoroReporter® FITC Protein Labeling kit (Molecular Probes, Eugene, OR). 40 μl of a 1 mM sodium bicarbonate buffer, pH 9, and 9 μl of a 10 mg/ml stock solution of reactive FITC dye in Me2SO were added to 400 μl of a 24 μM solution of hHsp90. The reaction was protected from light and incubated at room temperature for 1 h. Removal of free FITC dye was achieved by loading the reaction mixture on spin columns (provided with the kit) and centrifuging for 5 min at 1100 × g. The yield was a 17 μM solution of FITC-labeled hHsp90. The yHsp90 fragments were labeled accordingly.

**Electrophoretic Mobility Shift Assay—**DNA binding activity was an- alyzed by EMSA as described with minor modifications (12, 53). Briefly, specific complementary oligonucleotides containing the p53 consensus DNA binding site p21 (54) were fluorescence-labeled with a CY5-dye (MWG Biotech, Ebersberg, Germany) and annealed. Poly(dI-dC) (55) or pBluescript Vector (56) were used as unspecific competitors. The acti- vating and supershifting antibody Fab421 (Oncogene, San Diego, CA) was added to a final concentration of 100 ng per reaction (25 μl). Protein concentrations were 50 ng of p53 and 500 ng of hHsp90. yHsp90 frag- ments, p53-core, N313-p53, Hsp70, H4j1, GroEL/GroES, or Hsp27 per reaction. Samples were separated on 4% native polyacrylamide gels and quantified with a Typhoon 9200 Fluorescence Imager (Amersham Biosciences).

**Circular Dichroism—**Far-UV CD spectra were recorded in a J-715 spectropolarimeter with a PTC343 Peltier unit (Jasco, Germany) in 50 mM sodium phosphate, 50 mM sodium chloride, pH 7.5. The elipticity was recorded in 0.1-cm quartz cuvettes at 222 nm for 1 min to minimize signal noise. Thermal denaturation was followed via the change in elipticity at 222 nm from 10 °C to 90 °C with a rate of 20 °C/h. The spectra were corrected for buffer contributions.

**Fluorescence Anisotropy**—Cross-linking experiments were achieved under native conditions by addition of the heterofibrous zero-length cross-linker EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) (Pierce). In the respective experiments, p53 (1.5 μM) and EDC (5 mM) were incubated for 1 h at 22 °C in the presence or absence of hHsp90 (3 μM) in EDC reaction buffer (0.1 M MES, pH 5). The reaction was stopped by adding 10 mM ammonium acetate. Samples were sepa- rated on a 4–12% polyacrylamide gradient gel (Invitrogen) and stained with Coomassie Brilliant Blue (Serva, Heidelberg, Germany).

**Immunoprecipitation—**Immunoprecipitations were performed essen- tially as described (57) with purified p53 and hHsp90 at a volume of 100 μl. The reactions were incubated at 20 °C for 1 h in 50 mM sodium phosphate, 50 mM sodium chloride, pH 7.5, in the presence of the antibody Fab421, PAB240, or PAB1620 (Oncogene). Immunocomplexes were pelleted by binding to protein-A/G-Sepharose (Sigma). After three washes with 5 volumes of PBS buffer (4 mM potassium phosphate, 16 mM sodium phosphate, 120 mM sodium chloride, pH 7.4), the immu- nocomplexes were eluted with Laemmli SDS-loading buffer. For analysis, proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue (Serva).

**Nickel-chelating Magnetic Beads Assay—**For the qualitative analysis of the Hsp90-p53 interaction in solution, we used nickel-chelating mag- netic beads (NIMB, Qiagen). 340 pmol of FITC-labeled hHsp90 and 150 pmol of Histag-tagged p53 or p53 fragments were diluted at 4 °C in NIMB buffer containing 50 mM sodium chloride, 50 mM sodium phosphate, 20 mM imidazole, pH 7.5, to yield a total volume of 180 μl. The mixture was preincubated for 30 min at 10 °C. Subsequently, 20 μl of nickel-chelat- ing magnetic beads were added to the mixture, and the mixture was incu- bated for another 30 min at 10 °C. The beads were rinsed and vortexed three times with 500 μl of NIMB buffer. After resuspension in 100 μl of NIMB buffer they were analyzed with a Typhoon 9200 Fluorescence Imager (Amersham Biosciences, excitation 532 nm/emission 526 nm). The assay was carried out in the presence or absence of p53 and p53 fragments.

**Aggregation Assay—**p53 aggregates spontaneously at elevated temperatures. p53 (1.5 μM) or p53-core (1.5 μM) was incubated at 45 °C in the presence and absence of 15 μM hHsp90 after preincubation at 4 °C for 1 h in reaction buffer (50 mM sodium chloride, 50 mM sodium phosphate, pH 7.5). Assays in the presence of bovine serum albumin served as a control for unspecific protein effects. To monitor the kinetics of thermal aggregation, light scattering was measured in a FluoroMax II (Spx, Edison) fluorescence spectrophotometer in thermostatted quartz cuvettes. During the measurements, both the excitation and emission wavelengths were set to 340 nm with a spectral bandwidth of 5 nm.

**ELISA Assay—**For further analysis of the Hsp90-p53 binding we developed an ELISA-based protein interaction assay. 96-well high affi- nity microplates (Greiner, Kremsmünster, Austria) were coated with 50 μl of a 5 μg/ml solution of antibody PAbD01 (Oncogene) in PBS at 4 °C over night. PAbD01 recognizes residues 21–25 of the NTD of p53 specifically. The wells were rinsed with PBS at 4 °C three times. Blocking was done with 5% (w/v) milk powder (Roth, Karlsruhe, Germany) in PBS at 4 °C for 4 h. Following the blocking step, the wells were washed three times with PBS containing 0.01% (v/v) Tween 20 (Merck, Darmstadt, Germany). p53 (100 ng) or p53 fragments (100
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The tumor suppressor protein p53 consists of four functional domains (Fig. 1A). We constructed and purified six different p53 truncation fragments and the full-length protein to cover all domains of the human p53 protein in different combinations (Fig. 1A). The constructs correspond to the human wild type protein without mutations. The fragments p53-C363, p53-C212, N93-p53, and p53-core are able to bind specifically to the p21 DNA (data not shown). Size exclusion chromatography showed that wild type p53, p53-C363, N93-p53, and N313-p53 form tetramers, whereas the other fragments are in their monomeric state, due to the lacking tetramerization domain (data not shown). The correct secondary structure of all fragments was confirmed by CD spectroscopy (data not shown).

In Fig. 1B human Hsp90 (hHsp90), yeast Hsp90 (yHsp90), and the fragments used in this study are depicted. The structures and stabilities of the proteins were analyzed by CD spectroscopy and urea-induced unfolding transitions (42).

Temperature Dependence of the Stability and DNA-binding Activity of p53—Wild type p53 is a labile protein that is sensitive toward denaturation and aggregation. To analyze the correlation between the loss of secondary structure and the loss of DNA binding activity, we performed DNA binding assays (EMSAs) at increasing temperatures. The proteins were incubated for 15 min at the indicated temperature. Subsequently, the activating antibody PAb421 and p21 DNA were added at 4 °C (Fig. 2A). At incubation temperatures below 30 °C, identical results were obtained for the binding of p53 to p21 DNA. Above 30 °C, an irreversible loss of DNA binding activity was detectable, and the activity disappeared completely above 35 °C. At the physiological temperature of 37 °C, p53 was not able to bind to DNA any more. Activation of p53 by CKII-mediated phosphorylation of the RD gave similar results (data not shown). To analyze whether this loss in activity correlates with changes in the structure of p53, we monitored secondary structure by CD spectroscopy. These experiments showed that structural changes occur at temperatures above 25 °C, and a plateau was reached at 45 °C. The thermal unfolding of p53 is irreversible (data not shown). In Fig. 2B, the thermal denaturation of wild type p53, determined by CD spectroscopy, is compared with its DNA binding activity. These experiments showed that decreased DNA binding activity correlates with the loss of secondary structure.

Influence of Hsp90 on the Stability of p53—To test, whether the molecular chaperone Hsp90 has an influence on the inactivation process of p53, we performed activity assays in the presence of hHsp90 (Fig. 3A). Human Hsp90 itself did not exhibit binding affinity for the p21 DNA. As shown above, wild type p53 is not able to bind to p21 DNA after incubation for 15 min at 37 °C. However, addition of hHsp90 during the incubation at 37 °C resulted in the maintenance of DNA binding (Fig. 3A). At 4 °C, hHsp90 showed no influence on the activity of p53. Incubation with hHsp90 at 35 °C reveals a slight influence of hHsp90 on the activity, whereas at 40 °C, hHsp90 is not able to protect p53 any more. Addition of a higher excess of hHsp90 at 37 °C resulted in a protection of up to 100% in comparison to the DNA binding activity at 4 °C (data not shown). In the presence of hHsp90 no shift to a higher molecular mass of the DNA-protein complex signal after addition of hHsp90 was detectable, indicating that the interaction between p53 and Hsp90 is reversible.

In a similar experiment we tested whether this protective effect is specific for the molecular chaperone Hsp90 only. We used Hsp70, Hdj1 (Hsp40), Hsp27, and the GroE system in comparison to Hsp90 in the EMSA assay (Fig. 3B). All tested proteins had no effect on the DNA-binding ability of p53 at the thermal denaturation of p53.
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The squares activity. The thermal stability of the secondary structure of full-length was used to activate p53. The DNA-p53-antibody complex is shown. Fluorescence imaging of CY5-labeled p21 DNA. The antibody PAb421 ent temperatures and subjected to an EMSA. The gel was analyzed by
generation of p53. As shown in Fig. 3, Hsp90 is able to protect the DNA-
A 4 °C. As shown in Fig. 3A, Hsp90 is able to protect the DNA-
A 37 °C. Interestingly, neither Hsp70, Hdj1, Hsp27, nor the GroE system were able to preserve any p53 DNA binding at 37 °C, which leads to the conclusion that these chaperones are not able to protect p53 from heat inactivation in the EMSA assay.

It is known for many proteins, that unfolding often results in aggregation. To determine whether this is also true for p53, the protein was incubated at 45 °C and aggregation was measured by light scattering (Fig. 3C). Wild type p53 aggregated rapidly and reached a plateau after 5 min. Next, we investigated the influence of hHsp90 on the aggregation behavior of p53. Human Hsp90 is able to alter the aggregation kinetics in a distinctive manner, and it is also able to decrease the total p53 aggregation significantly. Human Hsp90 itself showed no aggregation at 45 °C (data not shown).

Folded Wild Type p53 Binds to Hsp90—To analyze the interaction of wild type p53 with Hsp90 in more detail, we employed several different experimental procedures (Fig. 4, A–D). Cross-linking of hHsp90 with p53 using EDC showed that p53 can be quantitatively and specifically cross-linked with Hsp90 (Fig. 4A). The presence of p53 and hHsp90 in the cross-link was confirmed by immunoblotting (data not shown). Interestingly, EDC is not able to cross-link p53 alone in its tetrameric state. This could be due to the high distance between the amino acids needed for the cross-linking-reaction (Arg, Glu, and Asp) in the tetramerization domain of p53.

Next, we performed immunoprecipitation of hHsp90 and wild type p53 at 25 °C using the p53-specific antibody PAB421, which recognizes residues 371–380 of human p53. It does not interact directly with hHsp90. However, in the presence of p53 the antibody was able to coimmunoprecipitate the two proteins (Fig. 4B). These experiments confirmed that Hsp90 binds to folded p53. To examine this interaction in more detail, we made use of two additional approaches. In an ELISA-based assay, the p53 antibody PAbDo1 was coated to the well of an ELISA plate. This antibody binds specifically to residues 21–25 of p53. Human Hsp90 was labeled with the fluorescence dye FITC and applied to the well. The experiments were performed at 4 °C (Fig. 4C), 25 °C (data not shown), and 37 °C (data not shown) with comparable results. As shown in Fig. 4C, hHsp90 is only detectable when p53 is present.

In a similar assay, we took advantage of the amino-terminal His tag to bind p53 to nickel-chelating magnetic beads (NiMB). FITC-labeled hHsp90 was added to the test tube at 25 °C. Here again, only hHsp90 was detectable, when p53 was present in the solution (Fig. 4D).

In an additional experiment, we used CY5-labeled p21 DNA to the NiMB-bound p53 fragment C363. This fragment was used because of its ability to bind to p21 DNA without antibody activation (data not shown). Binding of p53-C363 to p21 DNA was still possible. This indicates that p53 is in its native state and active while bound to NiMB (Fig. 5A) or ELISA plates (data not shown). Immunoprecipitation of p53 with the conformation-specific antibodies PAB240 (specific for denatured p53 only (58)) or PAb1620 (specific for natively folded wild type p53 only (59)) also showed that over 98% of the used p53 preparation is natively folded (Fig. 5B). The third evidence that p53 is in its native state gave an ELISA-based experiment, in which p53 was coated to the wells. The conformation-specific antibodies PAB240 or PAb1620 were added, and only binding of the wild type-specific antibody PAb1620 to p53 was detectable after addition of a CY5-labeled anti-mouse antibody (Fig. 5C).

Determination of the Hsp90 Binding Site in p53—Using p53 fragments, we determined the location of the Hsp90 binding site within p53. To analyze the domain interaction in detail, we bound different p53 fragments to NiMB. Due to surface-exposed histidine residues, even the fragments without His6 tags, e.g. N93-p53 and p53-core, were able to bind specifically to the NiMB. FITC-labeled hHsp90 was added to the complexes. Analysis of the reactions revealed that hHsp90 did not interact directly with the beads. However, hHsp90 is able to bind to all p53 fragments containing the DBD. For constructs that lack the DBD, no interaction with hHsp90 could be detected (Fig. 6A). In a second approach, we analyzed the domain interaction with the ELISA-based system. The p53 fragments that contain the DBD, no interaction with hHsp90 could be detected (Fig. 6B). Here again, only fragments containing the DBD were able to interact with hHsp90. Thus, we conclude that Hsp90 binds specifically to a folded conformation of the DBD.

Accordingly, the isolated DBD should be able to influence the interaction between Hsp90 and wild type p53 as a competitor. To test this, we incubated wild type p53 and hHsp90 in the presence and absence of an excess of the DBD at different temperatures and analyzed the functional state of p53 by EMSA (Fig. 7A). p21 DNA was added in high excess over p53-core and wild type p53 to exclude that this ligand is limiting. The DBD itself is able to bind to p21 DNA at 4 °C and 37 °C without changes in activity (data not shown). When the experiments were performed at 4 °C, in the presence of DBD or hHsp90 and DBD, the DBD had no influence on the DNA binding activity of wild type p53. At higher temperatures, the presence of the DBD abolished the protective function of hHsp90 for wild type p53. This result is consistent with a
competition of the DBD and wild type p53 for binding to Hsp90. The fragments p53-C93 (data not shown) and N313-p53 did not compete with wild type p53 for hHsp90. In the ELISA-based assay, the DBD was also able to compete with full-length p53 for hHsp90 binding (data not shown).

Because full-length p53 aggregates due to unfolding at elevated temperatures as described above, we performed a similar aggregation experiment with the p53-core fragment at 45 °C. Fig. 7B shows that the DBD aggregates within 20 min. In the presence of hHsp90, aggregation is decreased to a large extent. Human Hsp90 did not aggregate and was still active at 45 °C (data not shown). This result is evidence that the DBD is the specific binding site for Hsp90 and that aggregation is suppressed by the specific Hsp90-DBD domain interaction.

**Determination of the p53 Binding Site in Hsp90**—The location of the substrate binding site in Hsp90 has been discussed controversially (60–63). To determine the p53-binding site in Hsp90 we used yHsp90 fragments (see Fig. 1B). Human Hsp90 and yHsp90 bind to the DBD with comparable affinity (data not shown). In the ELISA system, we immobilized p53-core and added FITC-labeled yHsp90 fragments to the solution (Fig. 8A). The fragment N272, comprising the ATPase domain of Hsp90 did not bind to the DBD. Interestingly, both N527 and 529C bind to the DBD. However, the binding of the fragment N527 seems to be stronger than the binding of 529C.

According to the results, the Hsp90 fragments capable of binding to p53 should influence the DNA binding activity of p53 similar to full-length Hsp90. To test this, we incubated wild type p53 and yHsp90 fragments at different temperatures and analyzed the functional state of p53 by EMSA (Fig. 8B). The yHsp90 fragments did not influence the DNA binding activity of full-length p53 at 4 °C (data not shown). At higher temperatures only full-length yHsp90 and the fragments N527 and 529C were able to protect p53. The fragment N272 showed no protection, again confirming the participation of the middle domain and the carboxy-terminal domain of Hsp90 in the interaction with p53.

To determine the affinity of Hsp90 for p53, we performed titrations using different combinations of reaction partners. These included full-length p53 and N527, p53-core and hHsp90, and finally p53-core and N527. As shown in Fig. 8C, all combinations gave almost identical binding curves. These data suggest that the differences between yeast and humans and the influence of the carboxy-terminal region of Hsp90 to the binding to p53 are negligible for the interaction with p53. The binding constant of Hsp90 for p53 could be determined to be $K_D = 0.95 \pm 0.14 \mu M$.
DISCUSSION

The stability and activity of the tumor suppressor protein p53 has a significant influence on the cellular defense against cancer. The molecular chaperone Hsp90 seems to be involved in this process. However, only little information about the interaction between the two proteins and the functional consequences is available.

It has been reported before that wild type p53 is a labile protein, comprising folded and unfolded domains that function in a synergistic manner (12, 20). The thermal unfolding of p53 results in an irreversible loss of DNA binding activity. Interestingly, isolated p53 is already inactivated at physiological temperatures, showing the need for stabilizing interactions in vivo. It is established that Hsp90 interacts with p53 mutants in vivo and that it also affects wild type p53, especially under stress conditions (27, 46). As shown in the accompanying study by Walerych and coworkers (64), Hsp90 specifically influences the DNA-binding and transcriptional activity of wild type p53 under physiological conditions in vivo.

An important question in this context was whether the interaction between the two proteins is based on specific contacts in the native or unfolded state of wild type p53. To address this we analyzed the binding of purified p53 and Hsp90 with EDC. SDS-PAGE analysis of the cross-link of purified hHsp90 and equal amounts of purified wild type p53. The specific heterobifunctional cross-linker EDC was used. B, immunoprecipitation of p53 and Hsp90 using the antibody PAb421. SDS-PAGE analysis of an immunoprecipitation of purified p53 and equal amounts of purified hHsp90 using the p53-specific antibody PAb421. Immune complexes were recovered by binding to protein-A/G Sepharose. C, binding of FITC-labeled Hsp90 to immobilized p53. The p53-specific antibody PABo1 was attached to the well of an ELISA plate. After that, FITC-labeled hHsp90 together with or without p53 was added to the well. The fluorescence of hHsp90 was detected by fluorescence imaging. D, binding of FITC-labeled Hsp90 to p53. Wild type p53 was attached to nickel-chelating magnetic beads (NiMB) via its amino-terminal His6 tag in the presence or absence of FITC-labeled hHsp90. The fluorescence of hHsp90 was detected by fluorescence imaging.

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An important question in this context was whether the interaction between the two proteins is based on specific contacts in the native or unfolded state of wild type p53. To address this we analyzed the binding of purified p53 and Hsp90 using several independent methods. Cross-linking, immunoprecipitation, ELISA, and NiMB assays proved consistently that wild type p53 is able to bind to Hsp90 under conditions that favor the native state of p53. This suggests that a structured conformation of p53 is recognized. The finding that the p21 DNA can be bound by p53 in the presence of Hsp90 together with the finding that only the antibody specific for natively folded wild type p53 can recognize the protein further suggests that Hsp90 binds specifically to a native-like state of p53 that is in equilibrium with the active wild type conformation. The results of the EMSA assays finally show that binding of p53 to Hsp90 is reversible and that, after release from Hsp90, p53 is still able to bind to p21 DNA. This is in agreement with findings for citrate synthase, where Hsp90 binds transiently to highly structured
early unfolding intermediates, thereby preventing their aggregation and stabilizing the active species (65).

At heat shock temperatures, Hsp90 is able to compensate the irreversible loss of DNA binding activity of p53. This study and the accompanying study by Walerych and coworkers (64) show that, if Hsp90 is present at elevated temperatures, the folded conformation of p53 is protected and its binding activity to p21 DNA is preserved. Other chaperones, e.g. Hsp70, Hdj1, Hsp27, and the GroE system, are not able to compensate this loss of activity. The binding between Hsp90 and p53 is reversible, and p53 is in equilibrium with its active DNA binding form during binding to Hsp90. Furthermore, we could show that Hsp90 suppresses the aggregation of the full-length protein at these temperatures. The prevention of aggregation seems to be the consequence of protecting a folded, native-like conformation of p53.

Because other chaperones are known to interact with unfolded regions of a protein (66), the recently identified large unstructured regions in the amino-terminal region of p53 could represent the interaction site for Hsp90. The antibody PAb421 was used to activate p53. The DNA-p53-antibody complex is shown. B, influence of Hsp90 on the thermal aggregation of p53-core at 45 °C. The kinetic of aggregation was monitored by measuring the light scattering of the samples at 340 nm. p53-core (1.5 μM) was incubated in the absence (○) or presence of 15 μM (●) hHsp90. Human Hsp90 alone showed no aggregation at 45 °C (data not shown).
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Normalized data points were fitted using a hyperbolic regression analysis of the binding of increasing concentrations of FITC-labeled yHsp90-N527 to immobilized p53 (5 μM) in increasing concentrations (>). The fluorescence intensities were normalized. Data points were fitted using a hyperbolic regression function.

stability of the isolated DBD is significantly higher than the stability of the full-length protein (12). Thus, Hsp90 does not bind to an unstructured, but to a folded, native-like intermediate of the DBD. However, we cannot exclude that an unfolded DBD can, in principle, also interact with Hsp90 as suggested by others (49).

Until now there has been no agreement about a general binding site for substrates in Hsp90 (60–63, 67). This question had not been experimentally addressed for p53. Using truncation fragments of yeast Hsp90, we analyzed the interaction between wild type p53 and the domains of Hsp90. In the ELISA-based assay only full-length yHsp90, yHsp90-N527, and yHsp90–530C bind to p53, showing the possible existence of two binding sites for p53 within Hsp90. We could show that the binding to the middle domain seems to be significantly stronger than the binding to the carboxy-terminal domain of Hsp90, whereas the amino-terminal domain, which is proposed to bind to peptides (62, 63), did not bind to p53. Titrations of different combinations of fragments revealed that the affinity is determined by the interaction of Hsp90 with the DBD of p53. The binding constant of ~1 μM is in the range of substrate binding to Hsp70 (68). Interestingly, the middle domain and to some extent also the carboxy-terminal domain of Hsp90 are sufficient to protect p53 from thermal unfolding. Taken together, we suggest that Hsp90 is needed to ensure the functionality of wild type p53 at physiological temperature in vitro and in vivo, thus conferring an additional level of control for this key regulatory protein.

Acknowledgments—We thank the Zylicz laboratory (Warsaw, Poland) for sharing unpublished data and Titus M. Franzenmann and Sebastian Leptihn for helpful discussions. We gratefully acknowledge the experimental help of Christian Klein, Stefan Bell, and Roger Dawson. We thank Martin Hasbeck, Monika Ehrnsperger, and Stefan Walter for kindly providing the proteins Hsp27, Hdj1, GroEL, and GroES.

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