MDM2 Chaperones the p53 Tumor Suppressor*"S

Bartosz Wawrzynow†§, Alicja Zylicz†, Maura Wallace‡, Ted Hupp*, and Maciej Zylicz††

From the †International Institute of Molecular and Cell Biology in Warsaw, 02-109 Warsaw, Poland, ‡The Nencki Institute of Experimental Biology, PAS, 02-093 Warsaw, Poland, and the ¶University of Edinburgh, Division of Oncology, Cancer Research UK Cell Signalling Unit, Edinburgh EH4 2XU, Scotland, United Kingdom

The murine double minute (mdm2) gene encodes an E3 ubiquitin ligase that plays a key role in the degradation of p53 tumor suppressor protein. Nevertheless recent data highlight other p53-independent functions of MDM2. Given that MDM2 protein binds ATP, can interact with the Hsp90 chaperone, plays a role in the modulation of transcription factors and protection and activation of DNA polymerases, and is involved in ribosome assembly and nascent p53 protein biosynthesis, we have evaluated and found MDM2 protein to possess an intrinsic molecular chaperone activity. MDM2 can substitute for the Hsp90 molecular chaperone in promoting binding of p53 to the p21-derived promoter sequence. This reaction is driven by recycling of MDM2 from the p53 complex, triggered by binding of ATP to MDM2. The ATP binding mutant MDM2 protein (K454A) lacks the chaperone activity both in vivo and in vitro. Mdm2 cotransfected in the H1299 cell line with wild-type p53 stimulates efficient p53 folding in vivo but at the same time accelerates the degradation of p53. MDM2 in which one of the Zn2+ coordinating residues is mutated (C478S or C464A) blocks degradation but enhances folding of p53. This is the first demonstration that MDM2 possesses an intrinsic molecular chaperone activity, indicating that the ATP binding function of MDM2 can mediate its chaperone function toward the p53 tumor suppressor.

The p53 tumor suppressor gene encodes a sequence-specific transcription factor that is mutated in the vast majority of human cancers (1). Two other paralogs of p53, namely p63 and p73, have been identified, but the physiological functions of each member of the p53 family appear to be rather distinct (for review, see Refs. 2 and 3). One of the foremost characterized target genes of p53 is the mdm2 gene. MDM2 protein possesses E3 ubiquitin ligase activity toward p53 that plays a role in the negative regulation of p53 and its degradation by the proteasome (for review, see Ref. 4).

Through its ability to ubiquitylate p53 and target it for proteasomal degradation, MDM2 plays a key role in maintaining p53 at very low levels under non-stress conditions. In such circumstances MDM2 and p53 form a negative feedback loop in which p53 induces mdm2 transcription and MDM2 targets p53 for degradation (2). In stress situations, MDM2-dependent degradation of p53 is inhibited by a variety of mechanisms, including p14ARF binding to MDM2, phosphorylation of the C terminus of MDM2 by the ATM kinase, stress-induced phosphorylation of sites in the trans-activation domain of p53, subsequent binding of the p300 coactivator, and further acetylation of p53 in its C-terminal region (5), which results in an increase of the steady-state level of the p53 transcription factor and consequential flux in the expression of more than a hundred genes, including those involved in cell cycle arrest, senescence, and apoptosis (2).

Recent reports indicate that apart from its initially discovered RING finger-dependent enzymatic E3 ubiquitin ligase activity, MDM2 has other functions. A hydrophobic pocket in the N-terminal domain of MDM2 forms the basis for p53 transrepression. Small molecules that bind to this pocket can release p53 from MDM2-mediated transrepression (6). The MDM2 protein also possesses a nucleotide binding domain mediated by the consensus Walker P motif within the MDM2 RING domain that facilitates its nucleolar localization, but the ATP binding activity is not required for its E3 ubiquitin ligase activity (7). Allosteric effects within MDM2 protein involving its interaction with both the p53 transactivation motif and the p53 DNA binding domain can mediate p53 ubiquitylation (8). However, the collective roles of the RING domain, the ATP binding domain, and the hydrophobic pocket in the biological activity of MDM2 protein remain undefined.

MDM2 protein also interacts directly with the nascent p53 polypeptide (9), which suggests an unexpected role in protein biosynthesis. In the case of full-length p53, binding of MDM2 to an N-terminal sequence in p53 leads to its efficient proteasomal-dependent degradation. Surprisingly, it has been shown that the truncated version of p53, called p47, which lacks the p53 N-terminal MDM2 binding site, is expressed much more efficiently than full-length p53. It is proposed that this effect is not only due to decreased p47 degradation but also increased efficiency in the translation of p47 (9). A possible function in ribosomal biosynthesis or in translation regulation was suggested previously by specific interaction of MDM2 with the component of the large ribosomal subunit-L5 protein (10). MDM2 was also found to interact with several other ribosomal proteins like L11 and L23. Such binding that sequesters MDM2 inhibits p53 protein polyubiquitylation, resulting in its activation (11).
MDM2 Molecular Chaperone Activity

MDM2 oncoprotein also possesses numerous p53-independent activities, which contribute to the development of tumors where MDM2 is overexpressed, mostly by gene amplification possibly caused by polymorphism at the mdm2 promoter sequence (Ref. 12; for review, see also Refs. 13–15). Human cancers with non-functional p53 and amplification of mdm2 have poor prognosis (16). Transgenic mice with wild-type MDM2 genes are predisposed to spontaneous tumor formation in a p53−/− background and have a high incidence of lymphoma and sarcoma (17, 18). More than 40 MDM2 isoforms have been detected in human cancers (19). Interestingly, some of them, which do not possess a p53 binding domain, are associated with high grade and late-stage human cancer (20, 21).

The biochemical mechanism of oncogenic activity of MDM2 remains elusive. Indeed, although MDM2 interacts with proteins, fulfilling a variety of cellular functions (p300, pRb, Numb, MTBP (MDM2-binding protein), DNA polymerase ε, promyelocytic leukemia protein, Tip60, YY1, insulin-like growth factor 1 receptor, glucocorticoid receptor/estrogen receptor, androgen receptor, hypoxia-inducible factor 1, p73, NF-κB, PSD-95, ADP ribosylation factor, E2F1, TATA-binding protein, TAFII250, Sp1, ribosomal L5, TSG 101, Ras-GAP binding proteins), the biological significance of these multiple interactions remains to be explained (for review, see Refs. 15, 22). It should be stressed that not all MDM2 client proteins are targeted for proteasome-dependent degradation; hence, not all involvement of MDM2 protein can be explained by its E3 ubiquitin ligase activity (13, 15).

Several findings, namely binding to a nascent polypeptide chain (9), modulation of transcription factors (23–25), protection and activation of DNA polymerases (26), and involvement in ribosome assembly (10), indicate that the MDM2 protein possesses similar activities to those described for molecular chaperones (for review, see Ref. 27). Molecular chaperones are defined as a vast class of structurally unrelated proteins that assist in correct non-covalent assembly of other polypeptide-containing structures but which are not components of these assembled structures when they perform their normal biological functions (for review, see Ref. 28). Recently we have shown that the Hsp90 molecular chaperone, in an ATP-dependent reaction, retains p53 in a conformation that allows binding to a specific promoter sequence (29). Here we describe that, in the absence of polyubiquitlayment machinery, MDM2 can work synergistically with Hsp90, thus enhancing the binding of p53 to the promoter-derived sequence. Surprisingly, MDM2 alone possesses ATP-dependent molecular chaperone activity involved in folding of the p53 tumor suppressor protein. This is the first biochemical function attributed to the ATP-dependent domain of MDM2. We suggest that this newly discovered activity of MDM2 could not only play a role in p53 protein biosynthesis but could also in part explain the p53-independent oncogenic activity of MDM2.

MATERIALS AND METHODS

**Plasmid Preparation**—Human untagged MDM2 open reading frame lacking the first five codons (amino acids 6–491) inserted into a PT7.7 vector was prepared as described previously (30–32). The PT7.7 MDM2 K454A plasmid was prepared by means of site-directed mutagenesis using the QuikChange™ XL site-directed mutagenesis kit from Stratagene. For mutagenesis the following primers were used: mdm2K454A, GCATTGTC-CATGGCGCAACAGGACATC; mdm2K454Arev, GATGTC-GTTTGCATTGGCAATGTC; mdm2C478S, GGAATAAG-CCCTGGCAGTAAGCAGACACCAATTCTAAGTATT-GT; mdm2C478Srev, CAACAAGTCTTACATTGTGTTGTCT-TGCTTACTGGCCAGGGCTTATTCC; mdm2C464A, GGAC-ATCCTATGGCCCTTTACACGCGCAAAAGAGCTAAG-GAAAAG; mdm2C464Arev, CCTTTTCTTAGCTTTT-GCCGCTGTAAAGCAGGCCATAAGTAAGTC. The plasmids encoding MDM2 wt2 and the K454A or C478S mutant were used for purification of MDM2 wild-type and mutant proteins from *Escherichia coli*. Analogous mutations were introduced into the MDM2 wt open reading frame subcloned into the pCDNA3.1 vector.

**Expression and Purification of Recombinant Protein**—Human MDM2 wt was overexpressed in *E. coli* BL-21 RIL DE3 strain at 20 °C for 3 h after induction with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside. Cells were harvested by centrifugation at 8000 × g for 10 min and frozen in liquid nitrogen. Bacteria pellet was lysed in buffer A (100 mM Tris-HCl, pH 8.0, 200 mM KCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 5 mM Mg(CH₃COO)₂, 5 mM DTT, 1 mM benzamidine, protease inhibitor mixture EDTA free (Roche Applied Science), 1 tablet/50 ml of buffer A) containing 1 mg/ml lysozyme for 1.5 h at 4 °C with frequent stirring followed by 2 min at 37 °C and an additional 15 min at 4 °C. Afterward the suspension was centrifuged at 100,000 × g for 1 h at 4 °C. Under these lysis conditions most of the desired protein was insoluble and localized within the pellet after centrifugation. Extraction of the MDM2 protein from the cell pellet was carried out overnight at 4 °C with constant shaking. The following extraction buffer (B) was used: 25 mM Tris-HCl, pH 7.6, 1.2 mM KCl, 5 mM Mg(CH₃COO)₂, 1% Triton X-100, 5 mM DTT, 10% sucrose, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, protease inhibitor tablets. After centrifugation (100,000 × g for 1 h at 4 °C) the supernatant was collected and dialyzed into buffer C (25 mM HEPES-KOH, pH 7.3, 1 mM (NH₄)₂SO₄, 1 mM KCl, 5% glycerol, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride). After two h dialysis the sample was loaded onto a butyl-Sepharose column (Amersham Biosciences) equilibrated with the same buffer. The protein that bound to the column was eluted via gradient of decreasing ionic strength and increasing glycerol concentration. The fractions containing MDM2 protein were pooled and loaded onto a Q-Sepharose column equilibrated with buffer D (25 mM HEPES, pH 7.6, 50 mM KCl, 10% glycerol, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride). The flow-through from the column was immediately loaded onto a SP-Sepharose column equilibrated with an analogous buffer as the Q-Sepharose. The bound proteins to the SP-column were eluted by means of ionic strength gradient (from 50 to 800 mM KCl in buffer D). Fraction containing MDM2 protein were pooled, frozen in liquid nitro-
gen, and kept at –80 °C for further experiments. An analogous method was used to purify MDM2 K454A and MDM2 C478S from *E. coli*. Human recombinant p53 was purified essentially as described by Nichols and Matthews (33). Hsp90 proteins were purified as described in Walerych et al. (29).

**Enzyme-linked Immunosorbent Assay (ELISA)**—Investigation of the p53-MDM2 interaction was carried out using an ELISA technique. The method was performed analogously to the one described in Walerych et al. (29). For anchoring and/or detection of MDM2, monoclonal 2A-10 and 4B2 antibodies and polyclonal H-221 (Santa Cruz Biotechnology) were used, and for p53, monoclonal DO-1 and polyclonal FL-393 (Santa Cruz Biotechnology) were chosen. Inactivation of wt p53 was carried out at 37 °C for 90 min. Subsequently, 200 ng of heat-inactivated or correctly folded p53 (an aliquot of p53, reactive with the pAb1620 antibody, which was kept at 4 °C during the mentioned 37 °C incubation step) was coated onto ELISA plate wells in the coating buffer (25 mM Hepes, pH 7.3, 150 mM KCl, 10 mM DTT, for 1 h at 17 °C). After washing and blocking procedure with blocking buffer (25 mM Hepes, pH 7.3, 100 mM KCl, 2 mg/ml BSA), the anchored p53 was titrated with increasing amounts of MDM2 (preincubated or not with 5 mM ATP for 90 min) in the reaction buffer (25 mM HEPS, pH 7.3, 100 mM KCl, 15 mM MgCl2, 10 mM ZnSO4, 5% glycerol, 5 mM DTT, 0.1% Triton X-100, 2 mg/ml BSA) at 17 °C for 60 min. The experiment was also performed without Zn2+ ions, and no significant differences were observed.

**p53 DNA Binding Assay**—The DNA binding activity of p53 was quantified by EMSA (gel-shift) assay. The procedure of the assay was carried out as described in Walerych et al. (29). Minor modifications were introduced; that is, extension of the incubation time at 37 °C to 1.5 h, and with experiments that were based on a temperature gradient, a Biometra T Gradient Thermoblock was used. The samples were also supplemented not only with Hsp90 (bovine brain or human recombinant αHsp90 isoform) but also with human recombinant MDM2 wt, MDM2 K454A, or MDM2 C478S. Activation of p53 was predominantly carried out by 100 ng of the antibody pAb421 (Ab-1; Oncogene). However, control experiments were done with CK2 activation of p53. The specific p21 sequence was labeled, and the competitor unspecific DNA sequence was used as described in Walerych et al. (29).

**In Vitro p53 Ubiquitylation Assay**—100 ng of human recombinant p53 purified from *E. coli* was incubated in a 20-μl reaction volume containing 1× ubiquitination buffer (50 mM Tris pH 7.6, 100 mM KCl, 10 mM MgCl2, 2 mM DTT, 0.07 units of creatine kinase, 10 mM creatine phosphate, 5 mM ATP, 6 μg ubiquitin (human recombinant purified from *E. coli* or from Boston Biochem), 50 nM E1 ubiquitin activating enzyme (rabbit/human recombinant), 1.5 μM E2-conjugating enzyme UbcH5a (mouse/human recombinant), 3–150 nM E3 human recombinant MDM2 wt, MDM2 K454A, or MDM2 C478S. The reactions were incubated at 37 °C for 2 h. Afterward they were terminated with SDS sample buffer with β-mercaptoethanol, and the reaction products were fractioned by SDS-PAGE (8–10%). Transfer to a nitrocellulose/polyvinylidene difluoride film followed, and standard Western blot detection was carried out using anti-p53 DO-1 antibody.

**Tissue Culture Experiments**—Human lung carcinoma H1299 cells were grown at 37 °C in RPMI with 10% (v/v) fetal bovine serum, 5% CO2. Transient transfections were carried out as described in Dornan and Hupp (34). Cells were gently lysed on ice in Nonidet P-40 buffer (25 mM Hepes pH 7.5, 0.1% (v/v) Nonidet P-40, 150 mM KCl, 5 mM DTT, 50 mM NaF, protease inhibitor mixture by Roche Applied Science (1 tablet was added per 10 ml of lysis buffer)). The protein concentration of the cell lysates was quantified using the Bio-Rad Bradford assay kit. To estimate the amount of correctly folded p53 in H1299 p53−/− cell lines transfected with appropriate plasmids (pCDNA3.1 (EV)-mock, pCDNA3.1/p53wt, pCDNA3.1/MDM2wt, pCDNA3.1/MDM2K454A, pCDNA3.1/MDM2C464A, and pCDNA3.1/ MDM2C478S in appropriate combinations), the ratio between the level of p53 captured by pAb1620 and DO-1 was calculated. The ELISA procedure was carried out as follows: the wells were coated with wt-p53 conformation-specific pAb1620 monoclonal antibody or DO-1 (both mouse origin, Moravian Biotechnology) to 200 ng per well in 100 mM carbonate buffer, pH 9.0, at 4 °C for 16 h. The wells were blocked for 1 h at 17 °C with blocking buffer (4 mg/ml BSA in phosphate-buffered saline). This was followed by titration of increasing amounts of appropriate cell lysate diluted with the reaction buffer (25 mM HEPS, pH 7.3, 150 mM KCl, 5 mM DTT, 10% glycerol, 0.1% Triton X-100, 4 mg/ml BSA, protease inhibitor mixture). The reaction was performed for 1 h at 17 °C. Detection of p53 protein was carried out using the FL-393 antibody (rabbit origin, Santa Cruz Biotechnology) for 1 h at 17 °C. This was followed by the 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 kit (Bio-Rad) followed by quenching the reaction with 1 M H2SO4 and by absorbance measurements at 450 nm. The results obtained were plotted as a function of absorbance with respect to the amount of protein in question. For each particular case within the linear range the first derivative was calculated. The final values presented on the graph in Fig. 7 represent the normalization fractions obtained by division of pAb1620 derivative values versus DO-1 derivative values. The data represent the mean from five independent experiments with a standard deviation.

**RESULTS**

Recently we have shown that the Hsp90 molecular chaperone in an ATP-dependent reaction retains p53 at 37 °C in a conformation that allows binding to a specific promoter sequence (29). As shown by previously published data, p53 incubated at 17 °C *in vitro* binds specifically to promoter sequences after activation by C-terminal-specific p53 antibody or phosphorylation by CK2 kinase (Refs. 35 and 29 and Fig. 1A, lane 1). The same reaction performed under more physiological temperatures, i.e. 37 °C, does not lead to the formation of a p53 consensus site DNA complex (Fig. 1A, lane 2). As published before, at 37 °C p53 loses its correct fold, which is required for efficient specific binding of p53 to the promoter sequence (29). However, the ability of p53 to bind to a promoter sequence at 37 °C is possible in the presence of the Hsp90 molecular chaperone and ATP both *in vivo* and *in vitro* (Fig. 1A, lane 13, and...
Hsp90 and MDM2 may work synergistically. To test the effect of Zn$^{2+}$ on the binding of p53 to the p21 promoter-derived sequence, reactions containing 0.05 μg of p53 (58.1 nm of the monomer) and 0.6 μg of MDM2 (0.6 μM monomer), 10 μM ZnSO$_4$, and/or with 5 mM ATP. After activation of p53 by CK2, the DNA binding assay was performed. As shown in lane 3 the presence of zinc alone slightly stabilizes p53 at 37 °C; the effect is pronounced when Hsp90 is added (lane 5), but dramatic evidence of stabilization can be monitored when all three components are present together during heat inactivation of p53 (lane 7). Although both proteins, namely Hsp90 and MDM2, stabilize the wild-type conformation of p53 separately (compare lanes 2, 4, and 8), the zinc effect is more visible for the stabilization of p53 by Hsp90 than by MDM2 (compare lanes 4 and 5 and lanes 8 and 9). It is worth noting the additive effect of both proteins on the stabilizing effect of p53 during protein inactivation (lane 6).

The MDM2-dependent binding of p53 to the promoter sequence is limited to the physiological temperature of 37 °C (Fig. 3), suggesting that under heat shock conditions other chaperones could also be involved in these reactions. As shown in Figs. 1 and 2, the presence of MDM2 does not supershift the p53-promoter DNA complex, suggesting that MDM2 is only transiently required for p53 to adopt a DNA binding-competent conformation. To provide additional evidence that the interaction of MDM2 with p53 is indeed required for this effect, we used monoclonal antibodies that interfered with p53-MDM2 complex formation; 2A-10 binding to the central region of MDM2 and 4B2 antibody recognizing the epitope in the N-terminus of MDM2. The presence of these antibodies during the preincubation of MDM2 with p53 at 37 °C severely, in the case of 2A-10, and partially, in the case of 4B2, reduced the ability of p53 to bind to the promoter sequence (Fig. 4, lanes 4–6). Additional controls showed that anti-MDM2 antibodies, 2A-10 and to some extent also 4B-2, directly interfere with MDM2-p53 complex formation, as judged by using the ELISA approach described under “Materials and Methods” (result not shown). These data support previous findings that multidomains of MDM2 (at least the N-terminal domain and acidic domain) are involved in the functional interactions with p53 (8). Interestingly, the addition of the mentioned antibodies after

### FIGURE 2. The influence of zinc ions Zn$^{2+}$, Hsp90, and MDM2 on the binding of p53 to the p21 promoter derived sequence. Reactions containing 0.05 μg of p53 (58.1 nm of the monomer) were incubated for 1 h at 4 °C (lane 1) or for 1.5 h at 37 °C (lanes 2–9) without or in the presence of a constant amount of Hsp90 (bovine brain, 3 μM of the monomer), MDM2 (0.6 μM monomer), 10 μM ZnSO$_4$, and/or with 5 mM ATP. After activation of p53 by CK2, the DNA binding assay was performed. As seen in lane 3 the presence of zinc alone slightly stabilizes p53 at 37 °C; the effect is pronounced when Hsp90 is added (lane 5), but dramatic evidence of stabilization can be monitored when all three components are present together during heat inactivation of p53 (lane 7). Although both proteins, namely Hsp90 and MDM2, stabilize the wild-type conformation of p53 separately (compare lanes 2, 4, and 8), the zinc effect is more visible for the stabilization of p53 by Hsp90 than by MDM2 (compare lanes 4 and 5 and lanes 8 and 9). It is worth noting the additive effect of both proteins on the stabilizing effect of p53 during protein inactivation (lane 6).

### FIGURE 1. A, synergistic activity of MDM2 and Hsp90 in p53 binding to a specific DNA promoter-derived sequence at 37 °C. Reactions contained 0.05 μg of p53 (58.1 nm of the monomer) and were incubated for 1 h at 4 °C (lane 1) or 1.5 h at 37 °C (lanes 2–13) without or in the presence of a constant amount of Hsp90 (bovine brain) with or without 5 mM ATP and increasing amounts of MDM2 protein as indicated. To visualize the synergistic activity of Hsp90 and MDM2 protein, Hsp90 was added to the reaction mixture at 1.7 μM (of the monomer) final concentration, and under these conditions Hsp90 could only marginally restore the specific p53 binding to the promoter sequence (compare lane 3 to control lane 13 where the final concentration of Hsp90 was 1.7 and 3 μM, respectively). B, MDM2 restores p53-specific binding to the promoter sequence in a dose-dependent manner. The p53:MDM2 mass ratio for the reactions with increasing amounts of MDM2 (for increasing amounts of MDM2 protein as indicated. To visualize the synergistic activity of MDM2 and Hsp90 in p53 binding to a specific DNA promoter sequence in a dose-dependent manner. The p53:MDM2 mass ratio for the reactions with increasing amounts of MDM2 (for A and B alike) is 1:8 to 1:12, which is still significantly less than the amount of Hsp90 needed to perform a similar task. At a 1:12 protein mass ratio, 0.05 μg of p53 (58.1 nm of the monomer) and 0.6 μg of MDM2 (0.6 μM of the monomer) of the proteins was used. All shown EMSA bands in this and subsequent figures correspond to the size of the p53 tetramer bound to the DNA, shifted by the activating antibody pAb421.

Ref. 29 and 36). Surprisingly, the substitution of Hsp90 with MDM2 in this reaction leads to efficient binding of p53 to the promoter sequence (Fig. 1, A, lanes 8 and 11, and B, lanes 2 and 3). Moreover, the result presented in Fig. 1A (compare lanes 5 and 6 with and lanes 11 and 12, respectively) suggests that Hsp90 and MDM2 may work synergistically.

The experiments described in Fig. 1, A and B, were performed in the absence of exogenous Zn$^{2+}$. Zn$^{2+}$ ions are known to stabilize p53 and MDM2 structures, and the presence of Zn$^{2+}$ is required for Hsp90-MDM2 complex formation (37). To test the effect of Zn$^{2+}$, we performed p53 DNA binding experiments in the presence or absence of 10 μM ZnSO$_4$. As shown in Fig. 2, the presence of 10 μM Zn$^{2+}$ in the absence of any chaperones slightly stabilizes p53 at 37 °C, subsequently leading to binding to the p21 promoter in the EMSA assay (Fig. 2, lane 3). In the presence of Hsp90 and Zn$^{2+}$, visibly more p53 is bound to the promoter sequence at 37 °C (Fig. 2, lane 5). The addition of MDM2 increases p53 DNA binding activity measured in the presence of Zn$^{2+}$ and Hsp90 (Fig. 2, lane 7), but the effect of Zn$^{2+}$ is less pronounced (compare lanes 6 and 7 in Fig. 2). Zn$^{2+}$ ions do not significantly change the ability of MDM2 to rescue p53 binding to the promoter sequence at 37 °C (Fig. 2, lanes 8 and 9).

D. Walerych, unpublished results.
the activation step (activation by pAb421 (Fig. 4) or phosphorylation of p53 by CK2 kinase (result not shown)) do not inhibit the ability of p53 to bind to the promoter sequence (Fig. 4, lane 9 and 10), suggesting that on this stage MDM2 is no longer in the complex with p53. Moreover, the monoclonal MDM2-specific antibodies 4B-2 or 2A-10 do not change the mobility of p53-DNA complex which further supports the absence of MDM2 in the p53-DNA complex (Fig. 4, lane 9 and 10). However, if such a reaction is conducted at 4 °C, the monoclonal antibody 4B-2 supershifts the p53-DNA complex (Fig. 4, lane 14), suggesting that recycling of MDM2 from the p53 complex is caused by the diffusion accelerated by the temperature. The results presented in Fig. 4 suggest that the presence of MDM2 is required only when p53 is preincubated at 37 °C and that the p53-MDM2 complex dissociates after conversion of latent p53 to a transcriptionally active p53. In a control experiment (Fig. 4), we found that the addition of the p53-specific DO-1 antibody before (lane 8) and after preincubation of p53 at 37 °C (lane 12) leads to antibody-induced supershift, indicating that p53 is present in the complex with the promoter DNA sequence. In addition, using an ELISA approach, we have shown that DO-1 does not interfere with MDM2-p53 complex formation (see Fig. 6A and results not shown).

In the absence of ATP, MDM2 interacts with p53 independently of the conformational state in which p53 is, namely, a correct fold i.e. native-like or mutant-like fold (pre-incubation of p53 at 37 °C for 90 min, Fig. 5). This situation, however, is quite different in the presence of ATP. The nucleotide does not significantly influence the affinity of MDM2 to p53 with the correct wild-type conformation but has an effect when the wild-type conformation of p53 is abrogated. Here the presence of ATP leads to a reduction in the affinity of MDM2 toward p53 (Fig. 5A).
As reported previously, MDM2 possesses a Walker motif allowing it to preferentially bind ATP with $K_D = 13.5 \mu M$ (7). No ATPase activity of MDM2 was detected. Mutation of the $mdm2$ gene in the K454A position inhibits ATP binding by the protein but does not interfere with its E3 ubiquitin ligase activity (7). As shown in Fig. 5B and 6A, the purified mutant MDM2 K454 tightly binds p53 regardless of the presence of ATP or the conformational status of p53. These experiments suggest that binding of ATP by MDM2 triggers the release of MDM2 from the MDM2-p53 complex only when the wild-type conformation of p53 is abrogated. At the same time the MDM2 K454A protein, which still possesses E3 ubiquitin ligase activity on a level comparable with wild-type MDM2 (Fig. 6B), is not able to rescue p53 binding to the promoter sequence at 37 °C (Fig. 6C). Because the MDM2 K454A mutation is within the RING finger domain of MDM2, we cannot exclude that this mutation not only affects the ability of MDM2 to bind ATP but in addition has another effect(s) on the overall structure of the RING finger domain. However, two experimental facts strongly suggest that this is not a case. First, the MDM2 K454A mutant possesses E3 ubiquitin ligase activity similar to MDM2 wt protein (Fig. 6B). Kinetic studies show that MDM2 K454A is slightly more active in ubiquitylation of p53. Second, the point mutants MDM2 C464A and C478S in the zinc binding motif that are completely inactive in the p53 ubiquitylation reaction still efficiently bind ATP and are able to rescue the ability of binding of p53 to the promoter DNA sequence at 37 °C (result not shown).

The data presented in Figs. 5 and 6 suggest that recycling of MDM2 from the p53 complex, triggered by binding of ATP to MDM2, is required for binding of p53 to the promoter sequence at 37 °C. One explanation of these results could be that the binding of MDM2 to latent p53 partially unfolds p53, and after activation of p53 and ATP-dependent dissociation of MDM2 from the p53-MDM2 complex, p53 spontaneously folds to the correct conformation, allowing its binding to the promoter DNA sequence.

To test the influence of MDM2 on correct folding of p53 within the cell, we used an immunochemical-based assay that measures p53 affinity to conformation-specific antibodies (38, 39). Human lung carcinoma H1299 $p53^{+/−}$ cells, which express a limited amount of MDM2 (result not shown), were cotransfected with a combination of plasmids encoding wild-type p53 and wild-type or mutant proteins, MDM2 K454A, MDM2 C464A, and MDM2 C478S. After gentle cell lysis, the ratio of wild-type correctly folded p53 (ELISA test with pAb1620) to total p53 (ELISA test with DO-1) in cell lysate was measured (Fig. 7). In the control experiments we show that binding of the DO-1 antibody does not interfere with the p53-MDM2 complex formation (Figs. 4 and 5 and result not shown). Transfection of H1299 cells with $p53$ alone shows that less than 40% of p53 protein possesses a wild-type conformation (sensitive to pAb1620 monoclonal antibody). It is highly probable that in this case the correct folding of p53 is stimulated by the presence of other molecular chaperones, like the abundant Hsp90. Cotransfection of p53 wt with $mdm2$ wt substantially stimulates the folding of p53. More than 70% of p53 was found in a wild-type conformation. Cotransfection of p53 wt with

\[ 4 \text{ C. Stevens, S. Petterson, M. Wallace, B. Wawrzynow, A. Zylicz, M. Zylicz, and T. Hupp, manuscript in preparation.} \]
MDM2 C478S or C464A mutants, which are deficient in E3 ubiquitin ligase activity, caused an even higher level of pAb1620-recognizable conformation of p53 (Fig. 7). It must be stressed that in the case when \textit{mdm2} wt is used, we are dealing with at least three effects; that is, an increase of the degradation of p53 (result not shown), an increase of the chaperone-like activity of MDM2, and folding of wt p53 catalyzed by other chaperones like Hsp90. Cotransfection of cells with plasmid-encoding wt p53 and mutant MDM2 K454A substantially reduces the amount of p53 found in

**FIGURE 6.** The ATP binding mutant of MDM2, which is able to bind and ubiquitylate p53, is defective in promoting p53 binding to the \textit{p21} promoter sequence. A, purified MDM2 K454A, an ATP binding mutant, specifically interacts with human recombinant p53 similarly to wt-MDM2. An ELISA was used to verify binding, where constant amounts of MDM2 wt or MDM2 K454A (0.1 \(\mu\)g) were anchored onto the well via the 2A-10 monoclonal antibody, and increasing amounts of p53 protein were added. The secondary polyclonal antibody FL-393 was used to detect the complex formation. In a control experiment the p53 protein (0.1 \(\mu\)g) was anchored via the p53 antibody (DO-1) onto the well, and increasing amounts of MDM2 wt or MDM2 K454A proteins were added. The secondary polyclonal antibody H-221 was used to detect the complex formation. A BSA titration was used as a negative control of the interaction. B, MDM2 K454A possesses E3 ubiquitin ligase activity comparable with MDM2 wt in the \textit{in vitro} ubiquitylation assay. The reaction was performed as indicated and visualized as described under “Materials and Methods.” C, p53 protein binding to the specific DNA promoter at 37°C, mediated by MDM2, is ATP-dependent. The MDM2 K454A mutant was not able to maintain p53 binding to the \textit{p21} promoter sequence at restrictive temperature relative to wild-type MDM2. The EMSA assay was carried out as described under “Materials and Methods.” As indicated, comparable amounts of the two proteins were used in the EMSA and polyubiquitylation assays.
DISCUSSION

p53 is a protein of complex conformational flexibility, and it is in equilibrium between different functional conformations. Strongly unfolded or misfolded forms of mutant p53 can function as gain-of-function oncogenes (40–42). Moreover, wild-type p53 tumor suppressor protein may adopt these different conformations either in response to various stress conditions (post-translational modifications or the direct effect of elevated temperature), binding to DNA, or the interacting proteins (for review, see Refs. 43 and 44). Some of these conformational states can be detected by p53 conformation-specific antibodies (pAb1620 and pAb240) (38, 45).

It has previously been proposed that wild-type p53 protein could be transiently associated with molecular chaperones (29, 43, 46, 47). In vitro wt p53 can interact with Hsp40 and Hsp70, but such a complex is dissociated in the presence of Hsp90 (46). Transient interaction of p53 with Hsp90 is required for p53 binding to its consensus promoter DNA sequence (29, 36). When p53 is in a complex with a promoter sequence, it is no longer in a complex with Hsp90. The presence of ATP is required for Hsp90-dependent binding of p53 to the promoter sequence at 37 °C. Whether Hsp90 can unfold p53 protein actively (analog to the Hsp100 unfoldase activity as described in Ref. 48) or by transient binding to the conformationally flexible regions of p53 is still an open question. In vivo FRET experiments support the notion that the Hsp90 chaperone transiently interacts with wild-type p53.3

The presence of geldanamycin, a specific Hsp90 inhibitor, abrogates the binding of p53 to the p21 promoter sequence as well as its transcriptional activity (29). Geldanamycin-mediated stimulation of degradation of both wt and 175H mutant of p53 in H1299 cells coexpressing CHIP (chaperone-associated E3 ubiquitin ligase) has been shown (47). Recently we reported that cooperation of Hsp90 with MDM2 and CHIP proteins stimulates unfolding of the native tetramer of p53 (37). At the same time the MDM2-dependent polyubiquitylation of mutant p53 is inhibited by Hsp90 (49).

In this paper we have shown that MDM2 alone, in an ATP-dependent reaction, can substitute for the Hsp90 molecular chaperone in promoting the binding of p53 to the p21 promoter sequence at 37 °C. The results presented here and those published by others suggest a possible scenario depicted in Fig. 8. After binding to MDM2, p53 protein undergoes partial unfolding (50, 51). In a stress situation, after activation by protein kinases (or by pAb421 in vitro), MDM2 dissociates from p53, thus allowing p53 to spontaneously fold to a conformation that has a high affinity toward binding to the promoter sequence. This chaperone-like reaction is ATP-dependent and can be uncoupled from the E3 ubiquitin ligase function of MDM2 (mutations C478S or C464A). The mutation within the mdr2 gene, which encodes the MDM2 K454A protein with deficient binding of ATP, prevents the proper folding of p53 in vitro and in vivo. Activation of p53 after DNA damage and subsequent dissociation of MDM2 occurs on chromatin (53). Whether polyubiquitylation and subsequent degradation of p53 also occurs on chromatin remains still an open question (66).
lished in vivo data strongly support the idea of dissociation of MDM2 after the activation of p53. Using a chromatin immuno-precipitation technique, White et al. (53) have shown that MDM2 localizes with latent p53 on the chromatin near p21(waf1) and mdm2 genes before but not after DNA damage. We suggest that a transient complex of p53 with MDM2 and Hsp90 molecular chaperones may be important for the decision of whether to activate or degrade p53 by employing a series of post-translational modifications, like phosphorylation, acetylation, ubiquitylation, sumoylation, neddylation, and others. The situation where eukaryotic molecular chaperones are involved in folding and degradation of protein is similar to prokaryotic Hsp100/Clp family members, whereas an ATPase subunit of the appropriate protease at the same time is a molecular chaperone and the specificity factor for the protein substrates degradation (Refs. 54–56; for review, see Ref. 57). It is highly probable that in eukaryotes the quality control process starts upstream of the 19 S proteasome subunit, namely when the E3 ligases demonstrate their activities and the decision to repair or degrade the protein substrates occurs. This apparent contradictory mode of MDM2 as a component of the degradation machinery (E3 ubiquitin ligase activity) and as molecular chaperone-like activity resembles a dual function of CHIP, a cochaperone/ubiquitin ligase that targets a broad range of chaperone substrates for proteasomal degradation (58–60). Interestingly, while this paper was under revision, the Cyr and co-workers reported that CHIP alone possesses a molecular chaperone activity (61).

In addition to the fact that MDM2 can assist in correct non-covalent assembly of the p53-promoter complex, where MDM2 is not a component of this assembled structure, MDM2 fulfills other molecular chaperone criteria. The purified MDM2 protein, in an ATP-independent reaction, can function like the Hsp90 chaperone in protecting citrate synthase and firefly luciferase from aggregation (supplemental data). The discovery that MDM2, besides the E3 ubiquitin ligase activity, also possesses a molecular chaperone activity involved in protein folding and protection from aggregation raises an important question concerning the biological relevance of MDM2 chaperone activity toward p53 and other protein substrates. The in vivo situation of the p53 case is very complex. We observe that MDM2 promotes p53 folding but at the same time MDM2 accelerates the degradation of p53. We propose that molecular chaperone activities of MDM2 in concert with Hsp90 are required for shifting the equilibrium of p53 conformation toward such a state(s) where either p53 is potent to bind to the promoter sequence, thus being transcriptionally active, or to be recognized by the ubiquitylation machinery. If these and probably other chaperones are not able to shift the p53 conformational equilibrium, then p53 is directed by Hsp40 to a multichaperone complex, p53-Hsp40-Hsp70-HOP-Hsp90 (46). Such a stable complex inhibits not only p53 degradation but also p53 transcriptional activity. The overlapping chaperone activities (Hsp90, MDM2, and probably others) make verification of this hypothesis rather difficult. Nevertheless, recent findings from other laboratories indicate that ubiquitylation and proteasomal machinery are directly involved in transcriptional regulation. Zhu et al. (62) have shown that both ubiquitylation and proteasomal functions are required for efficient transcription mediated by p53. They postulate that among E3 ubiquitin ligases driving p53 toward ubiquitylation and degradation, MDM2 (besides TOPORS) cannot be excluded as a co-activator for p53 transcriptional activity. Emerging evidence indicates that MDM2 can modulate the activity of other transcription factors (23–25). We have found that MDM2, in an ATP-dependent reaction, stimulates unfolding of E2F1/DP1 transcription factor.4 MDM2 has been proved to be a positive regulator of HIV transactivator Tat. MDM2 promotes ubiquitylation of Tat and enhances Tat-mediated transactivation (63). Furthermore, MDM2 has been shown to recruit to the estrogen receptor-α binding p52 promoter (64) and modulate p63, p73, and E2F1 transcriptional activities and protein levels (3, 24, 65).

We postulate that molecular chaperone activity of MDM2 described here could not only help to explain the mechanism of involvement of ubiquitin-proteasome system in p53-mediated transcription (62) but also could shed new light on p53-independent activities of MDM2 as an oncogene (15). The findings reported in this paper that a single point mutation MDM2 K454A selectively inhibits a molecular chaperone activity of MDM2 should help in verifications of these hypotheses.

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