The cellular level of the tumor suppressor p53 is tightly regulated through induced degradation via the ubiquitin/proteasome system. The ubiquitin ligase Mdm2 plays a pivotal role in stimulating p53 turnover. However, recently additional ubiquitin ligases have been identified that participate in the degradation of the tumor suppressor. Apparently, multiple degradation pathways are employed to ensure proper destruction of p53. Here we show that the chaperone-associated ubiquitin ligase CHIP is able to induce the proteasomal degradation of p53. CHIP-induced degradation was observed for mutant p53, which was previously shown to associate with the chaperones Hsc70 and Hsp90, and for the wild-type form of the tumor suppressor. Our data reveal that mutant and wild-type p53 transiently associate with molecular chaperones and can be diverted onto a degradation pathway through this association.

The p53 tumor suppressor has been termed “the guardian of the genome” (1). In normal cells p53 is present at low concentration. DNA damage and other stresses such as hypoxia cause an accumulation of p53, which leads to cell cycle arrest or apoptosis (2, 3). p53 acts as a transcription factor to activate target genes that are involved in these responses to prevent damaged cells from proliferating and passing mutations on to the next generation (4). Cells that lack functional p53 are unable to respond appropriately to stress and are prone to oncogenic transformation. In fact, missense mutations that inactivate p53 are found in ~50% of all human tumors making them the most frequent genetic alterations in cancer (5, 6).

p53 is regulated through a variety of posttranslational modifications, including phosphorylation, acetylation, and attachment of ubiquitin, the small ubiquitin-like modifier SUMO and the ubiquitin-like protein Nedd8 (4, 7, 8). Central to the regulation of p53 is the ubiquitin ligase Mdm2 (8–10). Ubiquitin ligases (E3(s)) provide specificity to ubiquitin conjugation as the ubiquitin-like protein Nedd8 (4, 7, 8). Central to the regulation of ubiquitin, the small ubiquitin-like modifier SUMO and modifications, including phosphorylation, acetylation, and attachment of ubiquitin, the small ubiquitin-like modifier SUMO and the ubiquitin-like protein Nedd8 (4, 7, 8). Central to the regulation of p53 is the ubiquitin ligase Mdm2 (8–10). Ubiquitin ligases (E3(s)) provide specificity to ubiquitin conjugation as they mediate the final step in the conjugation process, following the activation of ubiquitin by the E1 enzyme and its transfer onto a ubiquitin-conjugating (E2) enzyme (11). Mdm2 belongs to the RING finger E3s, which facilitate ubiquitylation by tethering the E2-ubiquitin complex to the substrate protein (12). Mdm2-mediated ubiquitylation targets p53 for degradation by the 26 S proteasome and is of central importance for establishing low p53 levels in normal cells (13, 14). The functional interplay between Mdm2 and p53 was elegantly demonstrated in gene knock-out studies, in which the embryonic lethality of mdm2 null mice was rescued by simultaneous deletion of the p53 gene (15, 16). Stress-induced phosphorylation of p53 attenuates the interaction with Mdm2, leading to stabilization and activation of the transcription factor (17). Intriguingly, Mdm2 is itself a transcriptional target of p53, which establishes a negative feedback loop to terminate p53-mediated stress responses (18). Additional mechanisms that regulate the Mdm2-p53 interplay include autoubiquitylation of Mdm2, the association of Mdm2 with diverse binding partners, and alterations of the intracellular localization of Mdm2 and p53 (8, 10, 19, 20). Furthermore, Mdm2 not only mediates ubiquitylation of p53 but can also conjugate Nedd8 to the tumor suppressor to inactivate its transcriptional activity (7).

Despite the central role of Mdm2 in proteasomal targeting of p53, additional ubiquitin ligases were recently shown to participate in the degradation of the tumor suppressor in normal cells, including p300, Pirh2, and COP1 (21–23). Although p300 seems to cooperate with Mdm2 during ubiquitylation, Pirh2 and COP1 trigger the destruction of p53 independent of Mdm2. Multiple degradation pathways apparently exist to maintain low levels of p53 in normal cells. It is unclear whether these degradation pathways are truly redundant or whether they are selectively engaged in p53 destruction dependent on cell lineage, developmental stage, or physiological situation. In any case, the complexity of p53 degradation may allow to integrate diverse signaling events through which p53 can be regulated.

We have recently identified a pathway for protein degradation in the mammalian cytoplasm and nucleus that involves a close cooperation of the molecular chaperones Hsc70 and Hsp90 with the ubiquitin-proteasome system (24). Of central importance on this degradation pathway is the chaperone-associated ubiquitin ligase CHIP (25). Through binding to the carboxyl termini of Hsc70 and Hsp90, CHIP mediates the ubiquitylation of chaperone-bound client proteins in conjunction with E2 enzymes of the Ubc4/5 family and induces client degradation by the 26 S proteasome (26–28). Affected chaperone clients can be broadly divided into two subclasses: (i) Hsc70- and Hsp90-associated signaling proteins, for example the glucocorticoid hormone receptor (26, 29) and the oncogenic receptor tyrosine kinase ErbB2 (30) and (ii) aggregation-prone proteins that are subjected to chaperone-assisted quality control, such as misfolded cystic fibrosis transmembrane conductance regulator (31, 32) and hyperphosphorylated tau (33, 34). However, the full range of cellular substrates of CHIP remains to be explored. Remarkably, mice that lack CHIP develop apoptosis in multiple organs after environment-
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**MATERIALS AND METHODS**

Purified Proteins and Antibodies—The following proteins were expressed recombinantly and purified as described previously: rat Hsc70, human Hsp40, human UbH5b, human CHIP, and wheat Hsp70 (25). Purified bovine ubiquitin was purchased from Sigma. For immunoblotting anti-p53 (DO-1; Oncogene Research Products, San Diego, CA), polyclonal anti-Hsc70 (F. U. Hartl, MPI for Biochemistry), monoclonal anti-Hsp70 (SPA-802; StressGen Biotechnologies, San Diego, CA), and anti-CHIP antibodies (25) were used.

Cell Culture and Transfection—H1299 and H11003 cells were grown in RPMI media (Sigma) supplemented with 10% fetal calf serum, penicillin, and streptomycin. Transfection of H1299 cells was performed using DOTAP liposomal transfection reagent according to the protocol of the manufacturer (Roche Diagnostics). U2OS cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum, penicillin, and streptomycin. For transfection of U2OS cells, Effectene transfection reagent (Qiagen, Valencia, CA) was used. Cell extracts were prepared 24 h post-transfection.

Degradation Assays—To analyze the degradation kinetics of p53, H1299 cells were seeded in six-well plates and were transfected with 0.1 μg of pRC/CMV-p53wt or 0.1 μg of pcDNA3.1-p53R175H and 1.2 μg of pcDNA3.1-CHIP as indicated. The total amount of added DNA was kept constant at 0.8 μg by the addition of pcDNA3.1-CHIP. Protein lysates were prepared at indicated time points after addition of cycloheximide (20 μg/ml). Cells were washed once with phosphate-buffered saline and lysed in 100 μl of lysis buffer (50 mM MOPS, pH 7.2, 100 mM KCl, and 1 mM phenylmethylsulfonyl fluoride. Samples were incubated for 2 h at 30 °C and then analyzed by SDS-PAGE and phosphorimaging.

Reporter Gene Assays—H1299 cells were seeded in six-well plates and were transfected with 0.6 μg of a firefly luciferase reporter gene plasmid (p53-3TA-Luc, BD Biosciences Clontech), 0.2 μg pRC/CMV-p53wt, and 1.2 μg pcDNA3.1-CHIP as indicated. The total amount of added DNA was kept constant at 2.5 μg/well by the addition of pcDNA3.1. U2OS cells were seeded in six-well plates and were transfected with 0.2 μg of pgluc-p53 and increasing amounts of pcDNA3.1-CHIP from 0 to 0.5 μg as indicated. The total amount of DNA was kept constant at 0.8 μg/well by the addition of pcDNA3.1. Cells were washed once with phosphate-buffered saline and lysed in 100 μl of lysis buffer (50 mM MOPS, pH 7.2, 100 mM KCl, 0.5% Tween 20) containing Complete protease inhibitor. The lysate was centrifuged at 20,000 × g for 30 min at 4 °C, and the supernatant was used as a soluble extract. 5 μl of the soluble extract were analyzed with luciferase assay reagent (Promega). Levels of p53 and CHIP were determined by SDS-PAGE and immunoblotting.

RNA Interference—Endogenous CHIP was depleted in U2OS cells using siRNA oligonucleotides (Dharmacon, Lafayette, CO). The oligonucleotide CHIP1 is directed against the sequence GAAGACGGCTGGAAGCGCACC. CHIP2 targets the sequence ACCAGGGGTGAGGGA of the human CHIP gene. Green fluorescent protein siRNA (GGCTAGCTCCAGGCGGCAC) served as the control. U2OS cells were seeded in six-well plates and were transfected twice at a 72-h interval with siRNA oligonucleotides using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. 72 h after the second transfection cells were washed once with phosphate-buffered saline and lysed in 100 μl of radiomimetic precipitation assay buffer supplemented with Complete protease inhibitor. The lysate was centrifuged at 20,000 × g for 30 min at 4 °C, and the supernatant was used as a soluble extract. Equal amounts of protein were separated by SDS-PAGE. Levels of CHIP and p53 were determined by immunoblotting.

RESULTS

p53R175H but Not Wild-type p53 Forms Stable Complexes with Hsc70 in H1299 Cells—To investigate a potential influence of the chaperone-associated ubiquitin ligase CHIP on the turnover of p53, the human lung cancer cell line H1299 was used. In an initial experiment we analyzed the association of wild-type p53 and the conformational mutant p53R175H with molecular chaperones.

**FIG. 1.** p53R175H forms stable complexes with Hsc/Hsp70 in contrast to wild-type p53. H1299 cells were transiently transfected with pRC/CMV-p53wt (p53) and pcDNA3.1-p53R175H (p53R175H) as indicated. Control cells were untransfected. For immunoprecipitation a specific anti-p53 antibody (clone 6H7) was used. After SDS-PAGE and immunoblotting p53-associated Hsc/Hsp70 was detected using a polyclonal anti-Hsc/Hsp70 antibody. To monitor expression levels, 25 μg of protein extracts (ex.) were loaded.
radiation of p53R175H induced by the CHIP ubiquitin ligase (Fig. 2, B and C). Apparently, the oncogenic mutant protein can be turned over by a chaperone-assisted degradation pathway.

Despite the fact that complexes between wild-type p53 and Hsc70 were not detectable by immunoprecipitation, it was previously speculated that wild-type p53 may undergo highly transient interactions with molecular chaperones (39). We reasoned that such transient interactions might become detectable upon CHIP overexpression, when even those proteins that associate with Hsc70 in a highly transient manner would be irreversibly diverted onto a degradation pathway (29, 31, 32). Indeed, CHIP was able to induce the degradation of wild-type p53, albeit with a slightly reduced efficiency when compared with the findings for p53R175H (Fig. 3). Our data reveal that wild-type p53 associates transiently with molecular chaperones and can be diverted onto a degradation pathway through this association.

**Geldanamycin Induces the Degradation of Wild-type and Mutant p53 in H1299 Cells**—We investigated how the ansamycin antibiotic geldanamycin (GA) affects p53 degradation. GA specifically inhibits Hsp90, which usually results in the proteasomal degradation of client proteins that depend on the activity of the chaperone (52). The turnover of wild-type and mutant p53 was stimulated upon GA treatment, consistent with an association of both forms with Hsp90 (Fig. 4). However, only in the case of p53R175H was a synergistic effect of GA and CHIP elevation observed. The prolonged and more stable association of mutant p53 with Hsp90 and Hsc70 may provide the molecular basis for this synergism.

**CHIP Mediates Ubiquitylation of p53 and p33R175H in Cooperation with UbcH5b and Hsc70**—To verify that CHIP acts as an E3 ubiquitin ligase during p53 degradation, p53 and p53R175H were in vitro translated in rabbit reticulocyte lysate. Upon addition of purified CHIP to translation reactions, ubiquitylated forms of wild-type and mutant p53 accumulated (Fig. 5). An increase in the amount of ubiquitylated p53 was also observed when UbcH5b and Hsc70 were added, suggesting a close cooperation of CHIP, UbcH5b, and Hsc70 during the ubiquitylation of p53, similar to recent observations for other CHIP substrates (26, 32).

**CHIP Affects p53-mediated Transcription**—We analyzed how CHIP influences p53-mediated transcription using a reporter construct that contains firefly luciferase under the control of a p53-response element. Overexpression of CHIP led to a significant reduction of luciferase activity in corresponding cell extracts (Fig. 6A). Conceivably, such a reduction may be caused by a CHIP-induced degradation of luciferase itself. However, it was previously shown that CHIP does not target luciferase for degradation but stimulates the folding of lucifer-
FIG. 6. CHIP influences p53-mediated transcription. A, H1299 cells were transiently transfected with a reporter plasmid expressing firefly luciferase under control of a p53 response element. Cotransfections were carried out using p53 and CHIP expression vectors as indicated. Protein lysates were prepared, and luciferase activity was measured. Activities are expressed relative to uninduced promoter activity, which was set to 1. The error bars represent the S.D. of three independent experiments. B, levels of p53 and CHIP were detected by immunoblotting using specific antibodies. 40 μg of total protein were loaded. Immunodetection of Hsc70/Hsp70 served as loading control. C, U2OS cells were transiently transfected with a reporter plasmid expressing firefly luciferase under control of a p53 response element. Increasing amounts of pcDNA/CHIP were cotransfected ranging from 0 to 0.5 μg DNA. Activities are expressed relative to p53-induced promoter activity, which was set to 100%.

Depletion of Endogenous CHIP Stabilizes p53—Endogenous levels of CHIP were depleted in U2OS cells following transfection with siRNAs (Fig. 7). Intriguingly, depletion of the chaperone-associated ubiquitin ligase caused a significant increase of p53 levels. The findings emphasize the role of chaperone-assisted degradation in maintaining low concentrations of p53 under physiological conditions.

DISCUSSION

Here we identify a novel degradation pathway for the tumor suppressor p53. This pathway is entered through a transient association of wild-type and mutant p53 with molecular chaperones and involves the chaperone-associated ubiquitin ligase CHIP. CHIP cooperates with E2 enzymes of the Ubc4/5 family to mediate the attachment of an ubiquitin-derived degradation signal to chaperone-bound p53, which leads to the proteasomal destruction of the tumor suppressor. CHIP-mediated degradation critically depends on the interaction of the ubiquitin ligase with the chaperones Hsc70 and Hsp90, which were shown to present chaperone clients to the ubiquitin ligase (26, 29, 31, 32). It has long been appreciated that oncogenic mutant forms of p53 associate with Hsc70 and Hsp90 in tissue culture cells and in tumor specimens (37, 39). Because of conformational alterations mutant p53s are retained in complexes with the two chaperones and with several of their regulatory cochaperones (39, 54). Therefore, oncogenic mutants are amenable to a regulation through chaperone-assisted degradation. A similar association with molecular chaperones was not yet unequivocally demonstrated for wild-type p53, as complexes between the tumor suppressor and Hsc70 or Hsp90 could not be isolated (39). It was speculated, however, that such complexes may escape detection because of their highly transient nature (39). Such transient associations with Hsc70 or Hsp90 may become detectable when the chaperone client is irreversibly diverted onto a degradation pathway through the action of the CHIP ubiquitin ligase. In fact, we observed that wild-type p53 is sensitive to an elevation of the cellular levels of the chaperone-associated ubiquitin ligase. Furthermore, depletion of endogenous CHIP stabilized wild-type p53 in U2OS cells. Taken together, our data establish a role of molecular chaperones in the regulation of wild-type p53.

Evidence suggests that chaperones associate with p53 at multiple stages and influence the oligomeric state, the nucleocytoplasmic transport and the transcriptional activity of the tumor suppressor (reviewed in Refs. 55–59). For example, Hsc70 was shown to sequester a mutant form of p53 in the cytoplasm by masking a nuclear localization signal present at the carboxyl terminus (56). p53 displays multiple binding sites for Hsc70, located in the amino-terminal transactivation domain, the DNA-binding core domain, and the carboxyl terminus (60–63). Binding sites within the core domain are recognized with particularly high affinity by Hsc70, but they appear to be buried within the native protein. On the other hand, the amino-terminal region of p53 seems to be natively unfolded, and the carboxyl terminus is relatively unstructured (64–66). Binding sites within these regions may remain accessible for Hsc70 when the protein is largely in a native conformation. Notably, studies using conformational antibodies indicate that even the core domain is a metastable structure that is easily perturbed upon treatment with chelating or oxidizing agents or by raising the temperature (49, 67). The core domain is also recognized by Hsp90 (57, 68), and Walerych et al. (58) recently demonstrated that Hsp90 is required to maintain the DNA binding activity of the core domain under physiological conditions. Apparently, p53 is a protein of large conformational flexibility and seems to be in a conformational equilibrium between native and less structured states. This flexibility may provide the means for complex intra- and intermolecular interactions and for the association of p53 with a multitude of regulatory proteins. The accompanying conformational changes are apparently assisted by Hsc70 and Hsp90. In this regard the extended chaperone interactions observed for mutant p53 seem to represent a pathologic exaggeration of physiologic interactions of wild-type p53 with the chaperone machinery.

Conceivably, the CHIP-mediated degradation pathway might be entered through an association of a chaperone client with either Hsc70 or Hsp90, as CHIP is able to bind both chaperones. Treatment of tissue culture cells with small molecular inhibitors of Hsp90, such as GA, has helped to verify
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CHIP can enter the heterocomplex and then further promote p53 tetramers (59). Because of its ability to bind Hsp90, CHIP can form a heterocomplex comprising Mdm2, p53, and Hsp90 (59, 69). In the heterocomplex the activity of Mdm2 to bind CHIP and regulate, an anti-apoptotic activity of CHIP was recently demonstrated based on the analysis of CHIP knock-out mice (35). Although the anti-apoptotic activity was largely attributed to the role of CHIP in the conformational regulation of the heat shock transcription factor, the ability of the chaperone-associated ubiquitin ligase to induce p53 degradation may contribute to this activity.

REFERENCES

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