MDM2 is a short-lived protein that regulates p53 degradation. We report here that transient coexpression of MDM2 and several p53 hotspot mutants resulted in stabilization and increased expression of MDM2. Ectopic expression of the mutant p53<sup>175H</sup> allele by recombinant adenovirus infection or stable transfection also stabilized endogenous MDM2 in p53-null cells. A panel of human tumor cell lines expressing different endogenous mutant p53 alleles also contained stabilized nuclear MDM2 at elevated levels when compared with p53-null cells. MDM2 was present in complexes with mutant p53 in tumor cells, and stabilization of MDM2 required direct binding to mutant p53. These results reveal a novel property of mutant p53 and a unique feature of tumors with p53 missense mutations. Accumulation of stable MDM2 may contribute to tumorigenesis through its p53-independent transforming functions.

The p53 tumor suppressor is a transcription factor that is mutated in over 50% of human tumors primarily because of missense mutations in the DNA binding domain (1). In normal cells, p53 is present at very low levels because of rapid degradation through the ubiquitin-dependent proteasome pathway. The MDM2 oncoprotein is an important regulator of p53 turnover. Expression of MDM2 is activated by p53 at the transcription level (2, 3). MDM2 binds to p53 and promotes its ubiquitination by acting as an ubiquitin E3 ligase (4–6). Gene knockout of MDM2 in mice causes early embryonic death, which can be rescued by further inactivation of p53 (7, 8). Therefore, MDM2 functions as a major negative feedback regulator to maintain p53 at low levels and protect cells from growth arrest and apoptosis induced by p53.

About 70–80% of human carcinomas with p53 point mutations in the core (DNA-binding) domain express mutant p53 protein at high levels (9, 10). Although a major consequence of p53 mutation is loss of its tumor suppressor function, maintenance of high level mutant p53 expression during tumor development suggests that it may also have positive effects on cell proliferation. Ectopic expression of several p53 mutants (175H, 248W, 273H, 281G) in p53-null cell lines can increase tumorigenic potential and drug resistance (11–13). Mutant p53 can also activate the c-myc promoter (281G) and overcome the mitotic spindle checkpoint in normal human fibroblasts (175H, 245D, Refs. 14, 15). These observations suggest that mutant p53 has gain-of-function properties that enhance cell transformation independent of wild-type p53. Mutational analysis suggests that amino acids 22 and 23 of p53, which are essential for p53 function (16), are important for the gain-of-function phenotype of the 281G mutant (16).

MDM2 is overexpressed in certain tumors with wild-type p53 (17), suggesting that it may contribute to tumor development by inactivation of p53. However, MDM2 also has p53-independent activities that may play a role in malignant transformation. Targeted overexpression of MDM2 in the breast epithelium of p53-null mice can induce abnormal cell proliferation and aneuploidy (18). Transgenic mice expressing 2–4-fold higher levels of MDM2 develop sarcomas at higher frequencies in p53-null backgrounds (19). MDM2 can interact with and inactivate the retinoblastoma tumor suppressor (pRb, Ref. 20). It can also modulate the activity, stability, and apoptotic function of E2F1/DP1 transcription factors (21–23). Furthermore, MDM2 can abrogate the growth arrest function of TGF-β in a p53-independent fashion (24). Therefore, MDM2 can enhance cell transformation by p53-dependent and -independent mechanisms.

MDM2 is a transcriptional target of p53 and is often overexpressed in tumors with wild-type p53 because of enhanced transcription and translation (2, 25). MDM2 overexpression also occurs frequently through gene amplification (26, 27). In contrast, the status and function of MDM2 in tumors with mutant p53 is less well characterized. In cells overexpressing MDM2 because of gene amplification, MDM2 is degraded rapidly with a half-life of about 30 min (28). In this report, we present direct evidence that MDM2 in tumor cells with high levels of mutant p53 (175H, 241F, 248W, 249S, 273H, 273C, and 280K) is stabilized by interaction with p53. Several experimental approaches demonstrate that overexpression of mutant p53 can prevent MDM2 degradation, resulting in the accumulation of MDM2 in tumor cells to moderate levels. These results suggest a novel mechanism of MDM2 stabilization and accumulation.

**MATERIALS AND METHODS**

**Cell Lines and Recombinant Viruses—** H1T1080 (fibrosarcoma, wt p53), SJSA (osteosarcoma, wt p53, MDM2 amplification), C33A (HPV-negative cervical carcinoma, p53<sup>273H-C</sup>) and DLD-1 (colon carcinoma, p53<sup>241F-P</sup>) were obtained from the ATCC. H1299 (lung carcinoma, p53null), Saos2 (osteosarcoma, p53null), MDA-MB-231 (breast carcinoma, p53<sup>200R-K</sup>), MDA-MB-435 (breast carcinoma, p53<sup>245D-F</sup>), and MDA468 (breast carcinoma, p53<sup>226S-F</sup>) were provided by Arnold J. Levine. Adenoviruses expressing wild-type and p53<sup>241F</sup> were provided by Drs. Jiuyuh Lin and Bert Vogelstein.

**Transfection and Virus Infection—** H1299 and Saos2 cells were transfected with the pCMV-neo-Bam vector expressing 175H mutant p53 or human MDM2 cDNA using the calcium phosphate precipitation method. G418-resistant colonies were selected and pooled for further...
analysis. In transient transfections, each 10-cm dish of H1299 cells was cotransfected with 5 μg of CMV1-MDM2 expression plasmid and 5 μg of mutant CMV-p53 expression plasmid. Expression of MDM2 and p53 were analyzed 36 h after transfection. MG132 was added to 40 μM for 5 h where indicated. Recombinant adenovirus expressing wild-type or mutant p53 were amplified using 293 cells. H1299 and Saos2 cells were infected with diluted crude viral lysate at 200 plaque-forming units/cell. MDM2 protein levels were determined 24 h after addition of viruses. To determine the half-life of MDM2, 75 μg/ml of cyclohexamide was added to the cultures, and samples were collected at different time points for Western blot analysis.

Western Blot Analysis and Immunoprecipitation—Cells were lysed in radioimmunoprecipitation assay buffer (1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) and 100–200 μg of protein were fractionated by SDS-polyacrylamide gel electrophoresis. MDM2 and p53 were detected by monoclonal antibody 3G9 (29) and DO-1. The filter was developed using ECL-plus reagent (Amersham Pharmacia Biotech). For immunoprecipitation-Western blot analysis, cells were lysed in lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride). Cell lysate (300–600 μg of protein) was immunoprecipitated using anti-p53 monoclonal antibody Pab421 or anti-MDM2 monoclonal antibody 2A9 and was washed with lysis buffer.

**Immunofluorescence Staining**—Cells cultured on chamber slides were fixed with acetone/methanol (1:1) for 3 min at room temperature, blocked with PBS, 10% normal goat serum (NGS) for 20 min, and incubated with anti-p53 Pab1801 hybridoma supernatant (1:10 dilution) or anti-MDM2 2A9 hybridoma supernatant (1:100 dilution) in PBS, 10% NGS for 2 h. The slides were washed with PBS, 0.1% Triton X-100; incubated with fluorescein isothiocyanate-labeled goat-anti-mouse IgG in PBS, 10% NGS for 1 h; washed with PBS, 0.1% Triton X-100; and mounted.

**RESULTS**

**Transient Expression of Mutant p53 Induces MDM2 Stabilization**—Transient cotransfection of wild-type or mutant p53 with MDM2 expression plasmids can result in degradation of p53 (4, 5). In our assay system, a significant excess of MDM2 plasmid (>5-fold of p53) was required to achieve p53 degradation (data not shown). After cotransfection of equal amounts of mutant p53 and MDM2 plasmids into p53-null H1299 cells, p53 was not degraded. However, the levels of MDM2 expression in such transfections were significantly increased (Fig. 1A, compare lane 3 with lanes 5, 7, 9, 13). Among five p53 hotspot mutants tested, the 175H mutant showed the strongest ability to increase MDM2 expression and the 273H mutant was the weakest. Quantitation by serial dilution showed that MDM2 expression was induced 8-fold by the 175H mutant and 3-fold by the 273H mutant (data not shown). Mutant p53 had no significant effect on the expression of cotransfected green fluorescent protein GFP (Fig. 1A), suggesting that the increase of MDM2 expression was not because of enhancement of transfection efficiency.

To determine whether the increase of MDM2 level was because of stabilization, cotransfected cells were treated with the proteasome inhibitor MG132. The ability of MG132 to induce MDM2 accumulation provides an indication of its degradation rate before treatment. The results show that MG132 caused less dramatic accumulation of MDM2 that was coexpressed with mutant p53 (Fig. 1A, compare lane 3 and lanes 4, 5, 6). Therefore, higher MDM2 levels observed in the cotransfection with mutant p53 may be caused by stabilization of MDM2. Mutant p53 in these experiments were also highly stable, indicating that they were not degraded by MDM2 at this expression ratio (Fig. 1A). In MDM2 and wild-type p53 control cotransfection, interpretation of the result was complicated by the fact that wild-type p53 may repress MDM2 plasmid tran-
result shows that both cell lines expressed similar levels of MDM2 mRNA (Fig. 2B). This suggests that a change in protein stability is responsible for increased MDM2 level in H1299-175H cells.

Next, the effect of transiently expressed mutant p53 on endogenous MDM2 level was examined. To achieve uniform expression of mutant p53 in H1299 cells similar to tumor cells with p53 mutations, the cells were infected with a recombinant adenovirus expressing p53<sup>175H</sup>. This approach can achieve expression of p53 in nearly 100% of the cells, as verified by immunofluorescence staining (data not shown). The level of MDM2 was significantly increased 24 h after infection with the 175H virus but not with a lacZ control virus (Fig. 2C).

To further confirm that the increase in MDM2 level after stable transfection of 175H virus or infection with the 175H virus was due to an increase in stability, the half-life of MDM2 was determined. H1299 cells were infected with adenoviruses expressing wild-type or p53<sup>175H</sup> mutant for 24 h, and MDM2 expression levels were determined by Western blot analysis. In H1299 cells stably transfected with mutant p53 or infected for 24 h with p53 adenoviruses were determined after cyclohexamide treatment. Because wild-type p53 virus induced high level MDM2 expression, protein loading was reduced (10%) to obtain a similar signal intensity.

Because increased MDM2 expression can also be caused by factors other than wild-type p53 (such as bFGF and activated ras, Refs. 30, 31), we directly tested whether MDM2 stabilization occurred in the mutant cell lines. Cells were treated with the proteasome inhibitor MG132 for 5 h, and the MDM2 levels
were detected by Western blot analysis. MG132 treatment of p53-null H1299 or cells with wild-type p53 resulted in a significant increase of MDM2 levels (Fig. 3B). This is consistent with rapid degradation of MDM2 in these cells by proteasomes. In contrast, MDM2 levels in tumor cells with mutant p53 were only weakly induced after MG132 treatment, suggesting that MDM2 was not rapidly degraded by proteasomes in these cells. Densitometry analysis of the films showed that the levels of MDM2 in p53-mutant cells were at least 4-fold higher than in the p53-null H1299 cells, which is consistent with the immunofluorescence staining results. The MDM2 mRNA levels of DLD1 and C33A cells were also similar to that of H1299 (Fig. 2B), suggesting that mutant p53 induces MDM2 accumulation without stimulating transcription of the MDM2 gene.

To further confirm the stabilization of MDM2 in mutant p53 cell lines, MDM2 degradation rates were examined after treatment with the protein synthesis inhibitor cycloheximide. The half-life of MDM2 in the p53-mutant DLD-1 cells was significantly longer than that in SJSA and HT1080 cells, which express wild-type p53 (Fig. 3C). The half-life of MDM2 in SJSA is ~0.5 h, whereas in DLD-1 cells it is over 2 h. Using this assay, we also observed stabilization of MDM2 in C33A, MDA468, MDA231, and T47D cells (data not shown). Therefore, MDM2 stabilization may be a common event in tumor cells with mutant p53.

Stabilization of MDM2 by Mutant p53 Requires Complex Formation—MDM2 interacts with the N-terminal domain of p53, and this interaction is not significantly affected by point mutations in the core (DNA binding) domain of p53 (29). To determine whether p53 core domain mutants induce MDM2 stabilization through complex formation, we observed that the effect of 175H virus on the stability of MDM2 deletion mutant was investigated. In a previous experiment, stable Saos2 cell lines were established that express full-length MDM2 or the p53 binding-deficient Δ1–50 mutant (29). These cells were infected with 175H virus, and the stability of full-length and Δ1–50 MDM2 mutant were determined. The results show that in contrast to full-length MDM2, the Δ1–50 mutant was not stabilized by 175H virus infection (Fig. 4A). In a transient transfection assay using H1299 cells, 175H also did not stabilize a Δ58–89 MDM2 mutant (also deficient for p53 binding, data not shown). In a reciprocal experiment, MDM2 binding-deficient p5322Q/23S-281G triple mutant (Fig. 1C) also failed to stabilize MDM2 in transient transfection of H1299 cells (Ref. 16, Fig. 4B). These results demonstrate that MDM2 stabilization by mutant p53 requires complex formation.

To confirm the presence of the MDM2 mutant p53 complex in cells, lysate from C33A and stable H1299–175H cells were analyzed by anti-p53 Pab421 immunoprecipitation followed by anti-MDM2 Western blotting. Significant amounts of MDM2 were coprecipitated with mutant p53 from the cell lysate (Fig. 4C). MDM2 in the H1299 lysate was not precipitated by p53 antibody, demonstrating the specificity of the coprecipitation assay. Therefore, stabilized MDM2 is present in complexes with mutant p53.

DISCUSSION

The results described above demonstrate that expression of p53 core domain mutants in tumor cells causes stabilization of MDM2. We found that all five p53 hotspot mutants (175H, 248W, 249S, 273H, 281G) showed the ability to stabilize MDM2 in transfection assays using H1299 lung tumor cells (although with different efficiencies). Furthermore, solid tumor cell lines carrying 241F, 266E, 273H, 273C, and 280K mutations also have stabilized MDM2. These mutations are located in the evolutionarily conserved regions of p53, which are targeted by most of the mutations found in human tumors (Fig. 1C). Recently, MDM2 stabilization was also observed in a panel of leukemia cell lines with mutant p53 (32). Therefore, it is possible that this is a rather general property of tumor cells with p53 core domain mutations. Our results suggest a mechanism of MDM2 stabilization in such cases. Mutant p53 is degraded by MDM2 if the latter is overexpressed, such as by transfection of exogenous MDM2 (4, 5). However, it is apparent that at the ratio of MDM2 and mutant p53 naturally reached in tumor cell lines, mutant p53 is not efficiently degraded by the endogenous MDM2 but instead causes stabilization of MDM2.

It should be emphasized that the ability of mutant p53 to cause MDM2 stabilization does not mean that tumors with p53 mutation will accumulate MDM2 to very high levels. Immunohistochemical staining of tumor samples has revealed cells that overexpress both MDM2 and mutant p53, but this is a relatively rare event (27, 33). Our results show that expression of mutant p53 in H1299 cells can increase MDM2 level by severalfold. Such a moderate increase probably will not be defined as overexpression in conventional immunohistochemistry staining of clinical samples. MDM2 expression levels in tumors will also be limited by its transcription and translation rates (both are low in the absence of wild-type p53) and the still unknown threshold of the MDM2/mutant p53 ratio that will trigger p53 degradation.

RING finger mutations of MDM2 showed that the ability of MDM2 to promote p53 degradation is tightly linked to its ability to promote self-ubiquitination and degradation (34). Therefore, MDM2 stabilization may be caused by loss of its ubiquitin ligase function and may be related to stabilization of mutant p53 in tumors. Because MDM2 stabilization requires direct binding to mutant p53, it is possible that mutant p53 inhibits MDM2 ubiquitination after forming a complex. Mutant p53 may have obtained this capability because of altered structure or interaction with other cellular factors, such as heat shock proteins (35). Such a mechanism would suggest that mutant p53 is stabilized in part because of its ability to inactivate low levels of MDM2. A model proposed by Midgley and Lane (36) suggests that mutant p53 is stable because of the loss of
of MDM2 induction. However, tumor cell lines express readily detectable amounts of MDM2 bound to mutant p53, which may be induced by other transcription mechanisms (31). Therefore, reduction in MDM2 transcription after p53 mutation as well as the ability of mutant p53 to neutralize the function of remaining MDM2 may both contribute to p53 stabilization.

Mutant p53 has been shown to exhibit gain-of-function properties in the absence of wild-type p53. Although the precise mechanism of this phenomenon is still not clear, transcription activation and the MDM2-binding domain have been implicated by an experiment using the 22Q/23S//281G mutant (16). Our results show that the p5322Q/2381/281G mutant is also deficient in MDM2 stabilization. Therefore, it is possible that MDM2 stabilization also contributes to the tumorigenic activity of mutant p53. MDM2 transgenic mouse experiments have shown that a moderate increase (2–4-fold) of MDM2 expression can significantly increase the incidence of sarcomas in a p53-null background (19). Therefore, moderate MDM2 accumulation caused by p53 mutation may have an effect on tumor proliferation. Further experiments will be needed to test this possibility.

Acknowledgment—We would like to thank Thomas Guadagno for critical reading of the manuscript.

REFERENCES
1. Hollstein, M., Rice, K., Greenblatt, M. S., Soussi, T., Fuchs, R., Saria, R., Smith-Sorensen, B., Montesano, R., and Harris, C. C. (1994) Nucleic Acids Res. 22, 3551–3555
2. Wu, X., Bayle, K. H., Olson, D., and Levine, A. J. (1993) Genes Dev. 7, 1126–1132
3. Barak, Y., Juven, T., Haffner, R., and Oren, M. (1993) EMBO J. 12, 461–468
4. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997) Nature 387, 296–299
5. Kubbutat, M. H. G., Jones, S. N., and Vousden, K. H. (1997) Nature 387, 299–303
6. Honda, R., Tanaka, H., and Yasuda, H. (1997) FEBS Lett. 420, 20–27
7. Oca Luna, R. M., Wagner, D. S., and Lozano, G. (1995) Nature 378, 203–206
8. Jones, S. N., Roe, A. E., Donehower, L. A., and Bradley, A. (1995) Nature 378, 206–208
9. Soong, R., Robbins, P. D., Dix, B. R., Grieu, F., Lim, B., Knowles, S., Williams, K. E., Turbett, G. R., House, A. K., and Iacopetta, B. J. (1996) Hum. Pathol. 27, 1050–1055
10. Cripps, K. R., Purdie, C. A., Carder, P. J., White, S., Komine, K., Bird, C. C., and Willie, A. H. (1994) Oncogene 9, 2739–2743
11. Dittrich, D., Pati, S., Zambetti, G., Chu, S., Terskay, A. K., Moore, M., Finlay, C., and Levine, A. J. (1993) Nat. Genet. 4, 43–46
12. Blundin, G., Levine, A. J., and Oren, M. (1999) Oncogene 18, 477–485
13. Huang, M., Low, J., Dorn, E., Pu, D., Pattengale, P., Yeargin, J., and Haas, M. (1994) Am. J. Pathol. 145, 702–714
14. Fraizer, M. W., He, X., Wang, J., Gu, Z., Cleveland, J. L., and Zambetti, G. P. (1998) Mol. Cell. Biol. 18, 3735–3743
15. Guajardo, A., Aldape, K., Konakakizewicz, K., and Tlsty, T. D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5166–5171
16. Lin, J., Terskay, A. K., and Levine, A. J. (1995) Oncogene 10, 2387–2390
17. Juven-Gershon, T., and Oren, M. (1999) Mol. Med. 5, 71–83
18. Lundgren, K., Montes de Oca, Luna, R., McNeill, Y. B., Eimerick, E. P., Spencer, B., Barfield, C. R., Lozano, G., Rosenberg, M. P., and Finlay, C. (1997) Genes Dev. 11, 714–725
19. Jones, S. N., Hancock, A. R., Vogel, H., Donehower, L. A., and Bradley, A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15608–15612
20. Xiao, Z., Chen, J., Levine, A. J., Mojtahedi, N., Xing, J., Sellers, W. R., and Livingston, D. M. (1995) Nature 375, 694–698
21. Martin, K., Trouche, D., Hagemeyer, C., Sorensen, T. S., La Thangue, N. B., and Kouzarides, T. (1995) Nature 375, 691–694
22. Loughran, O., and T. Thangue, N. B. (2000) Mol. Cell. Biol. 20, 2186–2197
23. Plattner, C., Sparks, A., and Lane, D. (1999) Mol. Cell. Biol. 19, 3704–3713
24. Sun, P., Dong, P., Dai, K., Hannon, G. J., and Beach, D. (1998) Science 282, 2270–2272
25. Landers, J. E., Haines, D. S., Strauss, J. F., and George, D. L. (1994) Oncogene 9, 2745–2750
26. Oliner, J. D., Kizner, K. W., Melitzer, P. S., George, D. L., and Vogelstein, B. (1992) Nature 358, 80–83
27. Cordon Cardo, C., Latres, E., Drobnjak, M., Oliva, M. R., Pollack, D., Woodruff, J. M., Marechal, V., Chu, J., Brennan, M. F., and Levine, A. J. (1994) Cancer Res. 54, 794–799
28. Olson, D. C., Marechal, V., Momand, J., Chen, J., Romocki, C., and Levine, A. J. (1993) Oncogene 2353–2360
29. Chen, J., Marechal, V., and Levine, A. J. (1993) Mol. Cell. Biol. 13, 4107–4114
30. Shaulian, E., Resnitzky, D., Shifman, O., Blondheim, G., Amsterdam, A., Yawen, A., and Orent, C. (1997) Oncogene 15, 2717–2725
31. Ries, S., Biederer, C., Woods, D., Shifman, O., Shirasawa, S., Sasazuki, T., Mcmahon, M., Oren, M., and Cormick, F. (2000) Cell 103, 321–330
32. Pan, Y., and Haines, D. S. (1999) Cancer Res. 59, 2064–2067
33. Endo, K., Ueda, T., Ohta, T., and Terada, T. (2000) Liver 20, 209–215
34. Fang, S., Jensen, J. P., Ludwig, R. L., Vouwen, K. H., and Weissman, A. M. (2000) J. Biol. Chem. 275, 8945–8951
35. Nagaoka, Y., Anan, T., Yoshida, T., Mizukami, T., Utsuy, A., Fujiwara, T., Kato, H., Saya, H., and Nakao, M. (1999) Oncogene 18, 6037–6049
36. Midgley, C. A., and Lane, D. P. (1997) Oncogene 15, 1179–1189
