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Mapping of the p53 and mdm-2 Interaction Domains

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The 90-kDa cellular protein encoded by the mouse *mdm-2* oncogene binds to the p53 protein in vivo and inhibits its transactivation function (J. Momand, G. P. Zambetti, D. C. Olson, D. George, and A. J. Levine, Cell 69:1237–1245, 1992). cDNA clones encoding the human homolog of the mdm-2 protein (also called hdm-2) were isolated from a HeLa cell cDNA library. A series of monoclonal antibodies have been generated against human mdm-2 protein, and the epitopes recognized by these antibodies have been mapped. By construction of a series of deletion mutants, the region of the mdm-2 protein that is critical for complex formation with the p53 protein has been mapped to the N-terminal portion of the human mdm-2 protein. Interestingly, a monoclonal antibody with an epitope located in this same region failed to immunoprecipitate the mdm-2–p53 complex and appeared to recognize only free mdm-2 protein. The domain of the p53 protein that is sufficient for interaction with human mdm-2 protein has been mapped to the N-terminal 52 amino acid residues of the p53 protein. This region contains the transactivation domain of p53, suggesting that mdm-2 may inhibit p53 function by disrupting its interaction with the general transcription machinery.

The p53 gene can function as a tumor suppressor in both human and mouse cells. The wild-type p53 protein inhibits oncogene-mediated transformation, while mutant forms of this protein no longer act as tumor suppressors (5, 8). Mutation at the p53 locus is the most frequent genetic change documented in human neoplasms (13, 25, 34). Furthermore, patients with Li-Fraumeni syndrome carry germ line mutations in the p53 gene in a heterozygous state and develop a variety of tumors at high frequencies and at young ages (28, 42). Similarly, mice homozygous for inactivated p53 alleles are also predisposed to the development of multiple malignancies (3). The p53 protein may also function as a regulator of the cell cycle (29). It appears to play an important role in inducing growth arrest at the G_1-S border of the cell cycle in the event of DNA damage (16, 21), therefore maintaining genome stability and reducing the risk of mutations (27, 49).

p53 has been shown to be a DNA binding protein with the ability to activate transcription through binding to specific DNA sequences adjacent to promoters (7, 17, 19, 50). This suggests that the p53 protein can interact directly or indirectly with proteins of the transcriptional machinery in the cell. p53 protein is also the target of several oncogene products of DNA tumor viruses such as simian virus 40 (SV40) T antigen (23, 26), the adenovirus E1B 55 K protein (37), and oncogenic human papillomavirus E6 proteins (46). These viral oncoproteins have evolved to inactivate the biochemical and biological activities of p53 through specific protein-protein interactions. Each of the virus-encoded oncogene products inhibits the ability of p53 proteins to function as transcription factors (1, 30, 48).

Recently, the p53 protein has been shown to bind to a 90-kDa cellular protein (12). Amino acid sequence analysis of this protein identified it as the product of the *mdm-2* oncogene (33). The *mdm-2* gene was originally isolated as an oncogene amplified on a mouse double-minute chromosome (2, 6). Overexpression of the *mdm-2* gene in BALB/c 3T3 cells confers tumorigenicity in nude mice. Overexpression of *mdm-2* also resulted in the inhibition of p53-mediated transcriptional activation (33). Amplification of the human *mdm-2* gene has also been observed in several types of sarcomas containing the wild-type p53 gene and protein (22, 35), suggesting that it is also involved in the development of neoplasias in humans. To study the role of *mdm-2* in human oncogenesis and its involvement in the p53 pathway, cDNA clones encoding the human homolog of the mdm-2 protein were isolated from a HeLa cDNA library. This communication reports the mapping of the regions of the human mdm-2 and p53 proteins that are involved in the formation of a protein-protein complex in vitro. A series of monoclonal antibodies that recognize the mdm-2 protein have been produced, and the epitopes that combine with these antibodies have been mapped to the mdm-2 protein. One of these monoclonal antibodies binds to the region where mdm-2 forms a complex with p53 protein, and this antibody will only react with mdm-2 protein that is not bound to p53 protein.

MATERIALS AND METHODS

Isolation of cDNA clones. A λgt11 cDNA library prepared from HeLa cells was screened with the mouse *mdm-2* cDNA as probe under reduced stringency. The cDNA inserts were isolated from positive phages and were subcloned into the Bluescript vector for further characterization. A full-length cDNA containing the entire coding region was reconstructed from two overlapping clones and was completely sequenced by using the method of Sanger et al. (36).

In vitro translation and complex formation. Plasmids containing cDNA coding sequences were linearized by using restriction enzyme sites downstream of the coding regions, and RNA was prepared with either T3 or T7 RNA polymerases. In vitro translation and radiolabeling with [^{35}S]methionine was carried out with rabbit reticulocyte lysates obtained from Promega or Stratagene as directed by the suppliers. Typically, 20 μl of freshly prepared hdm-2-programmed lysate and 10 μl of p53-programmed lysate were mixed and were incubated at 30°C for 30 min to allow the formation of a p53–hdm-2 complex. For the detection of hdm-2 mutants binding to p53, p53 was translated by using unlabeled amino acids, and 1 mM unlabeled methionine was added before coincubation with labeled hdm-2 lysates. This

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treatment prevents synthesis of labeled p53, thus reducing background and allowing detection of hdm-2 mutants with mobilities similar to that of p53.

Immunoprecipitation assays. For the immunoprecipitation assays, the in vitro translation lysates were diluted in 300 μl of lysis buffer (50 mM Tris-Cl [pH 8.0], 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40); 100 μl of PAb421 or 300 μl of anti-hdm-2 monoclonal hybridoma supernatants was added with 5 mg of protein A-Sepharose 4B (Sigma). The samples were incubated at 4°C for 2 to 4 h with head-to-head tumbling. The protein A beads were washed with SNNTE (5% sucrose, 5.0 mM Tris-Cl [pH 7.4], 5 mM EDTA, 0.5 M NaCl, 1% Nonidet P-40), and the bound proteins were boiled in sample buffer (10% glycerol, 2% sodium dodecyl sulfate [SDS], 0.02% bromophenol blue, 10% β-mercaptoethanol, 125 mM Tris-Cl [pH 6.8]) and then were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the gels were treated with 10% acetic acid and 30% methanol, and then the gels were treated with a solution containing 1 M sodium salicylate and 5% glycerol. The gels were dried and exposed against Kodak X-Omat film at −70°C for 16 h.

Binding of hdm-2 to GST-p53 fusion proteins. Binding of hdm-2 to glutathione *S*-transferase–p53 (GST-p53) fusion proteins was performed as follows. *Escherichia coli* transformed with GST-p53 fusion protein expression plasmids was grown to an optical density at 595 nm of 0.8, 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added, and the cells were grown for 3 h. The cells were then pelleted, resuspended in 10 ml of phosphate-buffered saline (PBS), and lysed by sonication, and the cellular debris was removed by centrifugation.

Glutathione-Sepharose 4B beads (Pharmacia) were washed with NETN buffer (20 mM Tris-Cl [pH 7.5], 100 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40) containing 0.5% nonfat milk. Twenty microliters (packed volume) of beads was incubated with 0.2 to 1.5 ml (dependent upon the yield of fusion proteins) of *E. coli* lysates for 30 min at 4°C. The beads were then washed with BII buffer (20 mM Tris-Cl [pH 7.5], 100 mM NaCl, 2 mM EDTA, 0.1% Nonidet P-40, 2 mM dithiothreitol, 0.05% bovine serum albumin, 5% glycerol) and were incubated with 20 μl of in vitro-translated, ^{35}S-labeled hdm-2 lysate for 1 h at 4°C. The beads were washed with 1/2 SNNTE (2.5% sucrose, 2.5 mM Tris-Cl [pH 7.4], 2.5 mM EDTA, 0.25 M NaCl, 1% Nonidet P-40), boiled in sample buffer, and analyzed by SDS-PAGE.

Generation of monoclonal antibodies against hdm-2. A cDNA clone obtained from the library screening contained the N-terminal region of the hdm-2 coding region truncated at the first methionine initiation codon. This cDNA was recombined with the full-length hdm-2 cDNA to obtain a coding region without leader sequence and the first methionine. This coding sequence was then inserted into the pQE11 vector (Qiagen) to obtain a complete open reading frame with six histidine residues fused to the N terminus of hdm-2. The expression plasmid was then introduced into *E. coli*, and the histidine–hdm-2 fusion protein was purified by Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen) column chromatography. The major protein species in the purified preparation has a mobility similar to that of in vitro-translated hdm-2.

BALB/c mice were immunized with the *E. coli*-produced hdm-2 protein. Hybridomas were prepared with standard procedures and were screened by enzyme-linked immunosorbent assay and immunoprecipitation of in vitro-translated hdm-2 protein. Stable clones were established by three rounds of cloning. The epitopes of these monoclonal antibodies were determined by their ability to precipitate the panel of in vitro-translated hdm-2 deletion mutants used for p53 binding domain mapping.

Construction of hdm-2 deletion mutants. For hdm-2 mutants 50-491, 62-491, 102-491, 102-383, and 102-339, restriction sites were used to delete upstream coding sequences, causing translation initiation from the first downstream in-frame methionine codon. Mutants 6-204, 6-339, and 102-339 were derived from partial cDNA clones obtained from the library screening. Mutants 6-383, 1-294, and 1-222 were obtained by truncation of the transcription template by using restriction sites, thus causing termination of transcription and translation at or before the terminus. Mutants 19-491, 34-491, 123-491, and 153-491 were generated by polymerase chain reaction (PCR) amplification with primers HDM-AA19 (GGGAATTCACCATGATTCCAGCTTCGGA), HDM-AA34 (GGGAATTCACCATGCTTTTGAAGTTATT), HDM-AA 123 (GGGAATTCACCATGAGTGAGAACAGGTG), and HDM-AA153 (GGGAATTCACCATGGTTTCTAGACCATC) in combination with T3 or T7 primers. Mutants Δ222-294, Δ155-230, Δ155-160, Δ135-294, Δ90-153, Δ90-122, Δ59-89, and Δ43-58 were generated by using restriction sites in combination with the N-terminal truncation clones created as described above. Mutants Δ116-294 and Δ102-294 were produced by PCR amplification with primers HDM-AA116 (CGCCGATATCGATGATTCCTGCTGATT) and HDM-A A102 (CGCCGATATCATGGTATATATTTC) in combination with T3 or T7 primers and then were reconstructed with existing deletion clones described above.

Construction of GST-p53 fusions. GST-p53 fusions containing residues 1 to 160, 160 to 393, 160 to 320, 320 to 393, and 1 to 393 were kindly provided by Thomas Shenk. Fusions containing residues 1 to 145, 1 to 82, 1 to 52, 18 to 52, 18 to 82, and 45 to 145 were created by PCR amplification with the following primers: SN-AA1 (CGGGATCCCCATG GAGGAGCCGCAG), SN-AA145 (CCGAATTCCACAGC TGCACAGGGC), SN-AA18 (CGGGATCCCATTTTCAGA CCTATGG), SN-AA45 (CGGGATCCCGGACGATATTGA ACA), SN-AA52 (CCGAATTCCATTGTTCAATATCGT), and SN-AA82 (CCGAATTCCAGGAGGGGGCTGGTG). The fragments were inserted into the pGEX3X vectors (40).

RESULTS

Isolation of human *mdm-2* cDNA. A λgt11 cDNA library constructed from HeLa cells was screened by using the mouse *mdm-2* cDNA (35a) under conditions of reduced stringency. A total of 14 positive clones were isolated, and the cDNA inserts were subcloned into the Bluescript vector for further analysis. Preliminary restriction mapping and partial sequencing showed that they represent partial clones for the human *mdm-2* cDNA (6). A full-length coding region was constructed from two overlapping cDNA clones and was sequenced. The DNA sequence of this cDNA clone, designated *hdm-2*, is similar to the published *hdm-2* sequence (35), with complete identity within the coding region and a few differences in the noncoding regions. The fact that these two cDNA clones were obtained from two very different sources (HeLa cells versus colon carcinoma) yet have identical coding sequences suggests that they may represent the wild-type *hdm-2* coding sequence or that a systematic mutation is present in different cancer cells.

hdm-2 protein binds specifically to p53. The full-length cDNA was cloned into a Bluescript vector and used for in vitro transcription with T7 RNA polymerase. In vitro translation of the RNA in rabbit reticulocyte lysate generated a

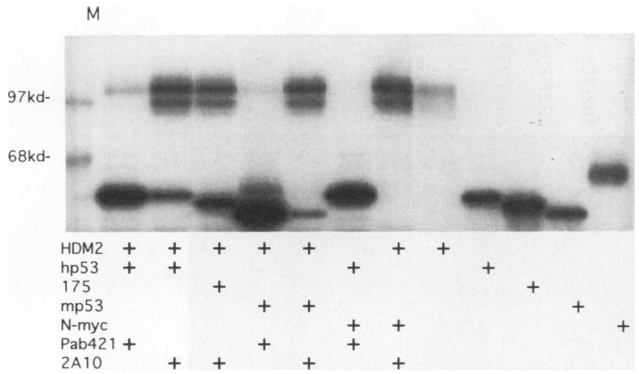

FIG. 1. Specific binding of hdm-2 to p53 in vitro. hdm-2, p53, and N-myc were translated and labeled individually with [^{35}S]methionine in rabbit reticulocyte lysates. They were mixed and incubated for 30 min, followed by immunoprecipitations with monoclonal antibodies against p53 (PAb421) or hdm-2 (2A10). hdm-2 and p53 can be coprecipitated with each other. N-myc does not coprecipitate with either hdm-2 or p53. The slower mobility of hdm-2 in some of the experiments is caused by the use of different batches of polyacrylamide solutions. M, molecular mass markers.

major protein product that is approximately 110 kDa in molecular mass by SDS-PAGE, as well as several minor species of lower molecular masses (97, 90, and 68 kDa) (Fig. 1). These minor products most likely represent initiation from internal methionines at residues 50 and 62 or premature termination of translation. The abnormally high apparent molecular weight of the hdm-2 protein was almost certainly caused by the acidic nature of the major part of the polypeptide. The full-length *hdm-2* coding region should produce a 491-amino-acid polypeptide with a calculated molecular mass of 54 kDa. It appears that the central acidic domain of *hdm-2* may be a major contributor to the abnormal mobility, because deletion of this region caused a significant decrease in the apparent molecular weight in SDS-PAGE analysis.

The rat mdm-2 protein was originally identified by its copurification with p53 in immunoprecipitation assays (12). To demonstrate the p53 binding activity of the hdm-2 protein, hdm-2 and p53 were each synthesized by in vitro translation and then were mixed and incubated at 30°C. Immunoprecipitations were then performed with either PAb421, an anti-p53 monoclonal antibody (10), or monoclonal antibodies against hdm-2. As shown in Fig. 1, hdm-2 can be coprecipitated or found in a complex with either human or mouse wild-type p53 protein. The human p53 mutant with an Arg-to-His mutation at codon 175 also bound to hdm-2 protein, consistent with the observation that several mutant human p53 proteins can bind to the rat p90 protein in vivo (12). As a negative control in the same assay, the mouse N-myc protein failed to bind to either hdm-2 or p53.

Deletion mapping of the p53 binding domain on the hdm-2 protein. A peptide sequence comparison between hdm-2 and other known p53 binding proteins, such as SV40 T antigen, human papillomavirus E6 protein, and adenovirus E1B protein, did not reveal any significant homology. To further characterize the interaction between the hdm-2 and p53 proteins, a deletion analysis of the hdm-2 protein was employed in order to determine the region of hdm-2 that interacts with p53. Three types of mutations were introduced into the hdm-2 cDNA: N-terminal deletions, C-terminal deletions, and internal in-frame deletions. These mutants were constructed either with convenient restriction sites to drop out a DNA fragment, employing PCR products to produce deletions, or with partial cDNA clones obtained from the library or combinations of these methods. Mutant cDNAs were then transcribed and translated in vitro. The [^{35}S]methionine-labeled hdm-2 proteins were then incubated with unlabeled p53 proteins made by in vitro translation and then were immunoprecipitated by using PAb421 antibodies directed against the p53. A panel of 24 hdm-2 protein deletion mutants were constructed and tested (see Fig. 3). The autoradiographs of the immunoprecipitates analyzed on SDS-polyacrylamide gels from one experiment are presented in Fig. 2. The results for the entire panel of mutants employed are presented in Fig. 3. Together, these hdm-2 mutants revealed that the region between amino acid residues 19 and 102 was critical for stable interaction with p53 protein in vitro. N-terminal deletions or internal deletions affecting part or all of this entire region result in significantly reduced binding to p53 protein (<5% of wild-type binding or undetectable). Clearly, three internal deletions affecting different portions of the protein between amino acids 19 and 102 all resulted in a loss of p53 binding. However, the smallest mutant that could bind to the p53 protein in this assay contained amino acids 1 to 294, which is significantly larger than the hdm-2–p53 binding region mapped by these internal deletions. In addition, the first 150 residues of hdm-2, when translated in vitro or synthesized in *E. coli* as a GST fusion protein, did not bind to p53 proteins in vitro (data not shown). All of these results then suggest that amino acid sequences outside of the region between residues 19 and 102 contribute structural elements that are required to form a functional p53 binding domain. Thus, the region from 19 to 102 appears to contain the site for p53 binding, but additional mdm-2 protein sequences which may be supplied by either region 102 to 294 or region 294 to 491 (Fig. 3) help to stabilize these p53–mdm-2 interactions. The regions 102 to 294 and 294 to 491 can each be deleted when the other is present.

Both the rat p90 protein and the hdm-2 protein are able to bind to p53 mutant proteins. To investigate whether there is any difference between the interaction of hdm-2 with mutant or wild-type p53 proteins, the binding between the hdm-2 mutants and a human p53 mutant protein (with mutation at codon 175) was examined. No differences between the binding patterns of hdm-2 mutants to wild-type p53 and the codon 175 mutant p53 protein were found (data not shown), indicating that mutant and wild-type p53 interact with the same region on hdm-2 and require a similar degree of integrity of this region.

An epitope map for hdm-2 monoclonal antibodies. A series of murine monoclonal antibodies were prepared by using the hdm-2 protein as an antigen. The *hdm-2* cDNA clone was inserted into a bacterial expression vector which fused six histidine residues to the N terminus of the full-length hdm-2 protein. The hdm-2 protein was then produced in bacteria and purified by a Ni-chelating column chromatography step. Antibodies made by a large number of monoclonal antibody cell lines were screened for their ability to react with hdm-2 and mdm-2 and the stable, high-titer, and specific antibody-producing cell lines were selected.

The availability of the mutant *hdm-2* cDNA clones (Fig. 3) permitted the mapping of epitopes on the hdm-2 protein by these hdm-2 monoclonal antibodies. The epitope map was constructed by noting the ability or failure of an antibody to immunoprecipitate wild-type or mutant hdm-2 protein. The map of these epitopes is presented in Fig. 4.

Monoclonal antibodies that recognize the epitopes in the hdm-2–p53 binding site. When these monoclonal antibodies

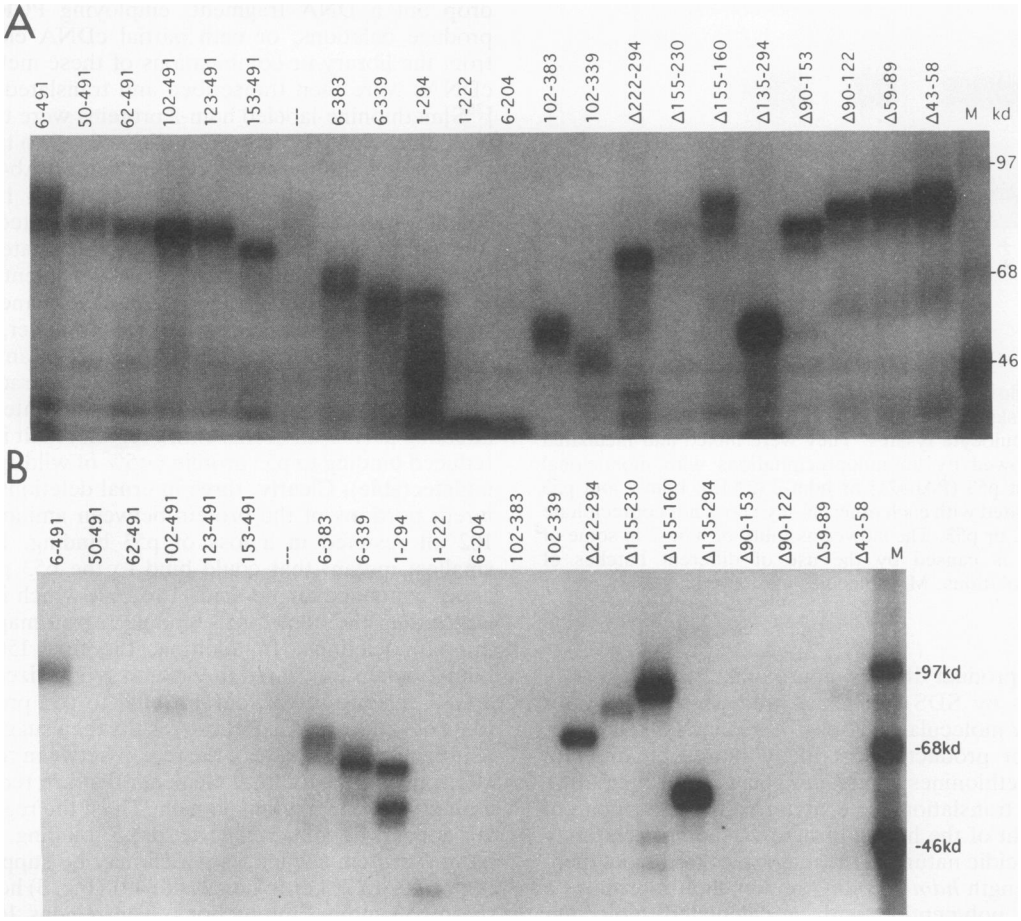

FIG. 2. (A) In vitro translation products of the hdm-2 deletion mutants. One microliter of the ^{35}S-labeled in vitro translation products was run on the SDS-polyacrylamide gel to verify the synthesis of mutant proteins. (B) Coprecipitation of hdm-2 mutants with p53. The labeled hdm-2 mutant proteins were incubated with in vitro-translated, unlabeled human p53, followed by immunoprecipitation with PAb421. The coprecipitated hdm-2 proteins were detected by SDS-PAGE and autofluorography. Mutants 6-491 (similar to the wild type) and Δ155-160 are not included in Fig. 3 in order to reduce redundancy. Four additional mutants, 19-491, 34-491, Δ116-294, and Δ102-294, were analyzed in separate experiments and the results are presented in Fig. 3 without these data being presented in Fig. 2. M (A and B), molecular mass markers.

were employed to immunoprecipitate the mixtures of hdm-2 and p53 proteins synthesized by in vitro translation, most of these antibodies were able to coimmunoprecipitate the hdm-2-bound p53 protein (data not presented). However, antibody 3G5, with an epitope located between amino acid residues 59 and 89 in the p53–hdm-2 binding site, could only immunoprecipitate free hdm-2 and failed to react with hdm-2–p53 complexes (see Fig. 5A). Furthermore, an immunoprecipitation with both 4B2 and 3G5 efficiently coprecipitated p53, suggesting that 3G5 was not able to dissociate preformed hdm-2–p53 complex. This same observation was made when the p53–mdm-2 protein complexes from cells in culture were used instead of in vitro translation products. The rat A-1 cell line contains the rat p53–mdm-2 protein complex and free rat mdm-2 protein (33). The monoclonal antibody 3G5 only immunoprecipitates the free rat mdm-2 protein and fails to detect the p53-bound rat mdm-2 protein (Fig. 5B). Other hdm-2 monoclonal antibodies can immunoprecipitate both free mdm-2 protein and mdm-2 protein bound to p53 protein (Fig. 5B and data not shown). This observation suggests that the in vitro association of p53 and hdm-2 proteins accurately reflects what is happening in a cell extract and a cell in vivo.

It is possible that the monoclonal antibody 3G5 that recognizes an epitope on the hdm-2 protein between amino acid residues 59 and 89 is physically blocked from binding to this epitope by the p53 protein that is bound to hdm-2. However, it remains possible that the region between residues 59 and 89 of this protein exists in two states (conformational or due to modifications): one that can bind to p53 and one that cannot bind to p53 protein. In this case, this monoclonal antibody would be specific for the non-binding epitope on hdm-2.

Mapping of hdm-2 binding domain on the p53 protein. It has been demonstrated that overexpression of mdm-2 results in the inhibition of p53-mediated transcription activation. Because this modulation of p53 activity is likely mediated by the binding of mdm-2 to p53, identifying the region of p53 that interacts with mdm-2 should provide information about the possible mechanism of this regulatory activity. The functions and tertiary structure of p53 are known to be sensitive to point mutations and deletions (4, 9, 17, 18). However, the ability of different p53 missense mutants to bind to mdm-2 (12) indicates that this protein-protein interaction is less sensitive to these conformational changes of

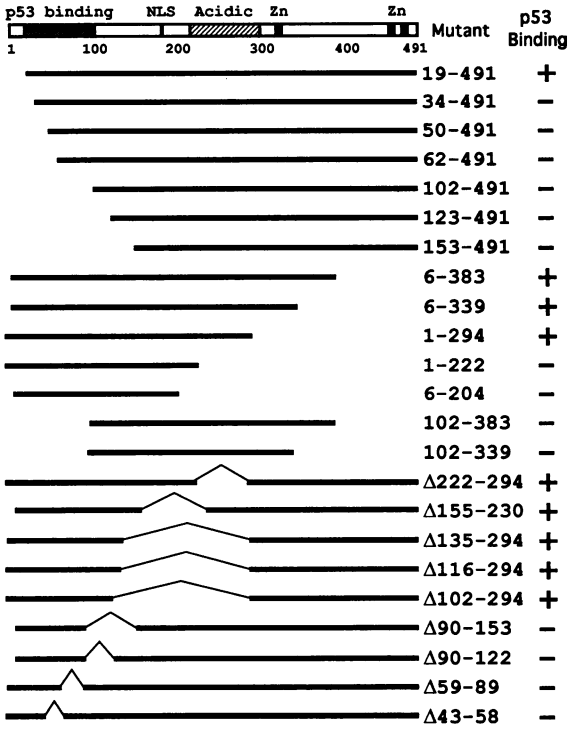

FIG. 3. Diagrams of hdm-2 deletion mutants and summary of p53 binding activity. The top open bar represents the full-length hdm-2 protein and the locations of the possible functional motifs recognizable by sequence analysis. The thick solid bars represent the region of hdm-2 polypeptide encoded by each mutant. The bent thin lines indicate the regions deleted in the internal deletion mutants. The numbers representing each mutant also indicate the amino acid residue numbers at the boundaries of the deletions. Mutants with undetectable binding or <5% of binding efficiency compared with full-length hdm-2, as measured by the amount of bound versus input protein, are considered negative. The region critical for p53 binding can be located between residues 19 and 102. During sequence examination, an additional C2H2 type zinc finger motif was noticed between residues 438 and 457, in tandem with the previously identified C-terminal zinc finger. NLS, nuclear localization signal; Acidic, acidic region; Zn, zinc finger.

p53. Therefore, GST-p53 fusions were employed to locate the hdm-2 binding region on the p53 protein.

Different portions of human p53 were fused to the carboxyl terminus of the GST protein and were used to test for

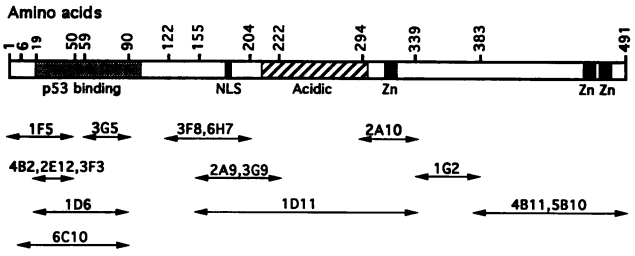

FIG. 4. Epitope map of the anti-hdm-2 monoclonal antibodies. The open bar represents the hdm-2 polypeptide, and the structural motifs are indicated. The double-arrowed lines indicate the regions that contain the epitopes for each antibody. The amino acid residue numbers for the boundaries of each region are shown above the bar. It has not been determined whether the antibodies which mapped to the same regions recognize identical epitopes.

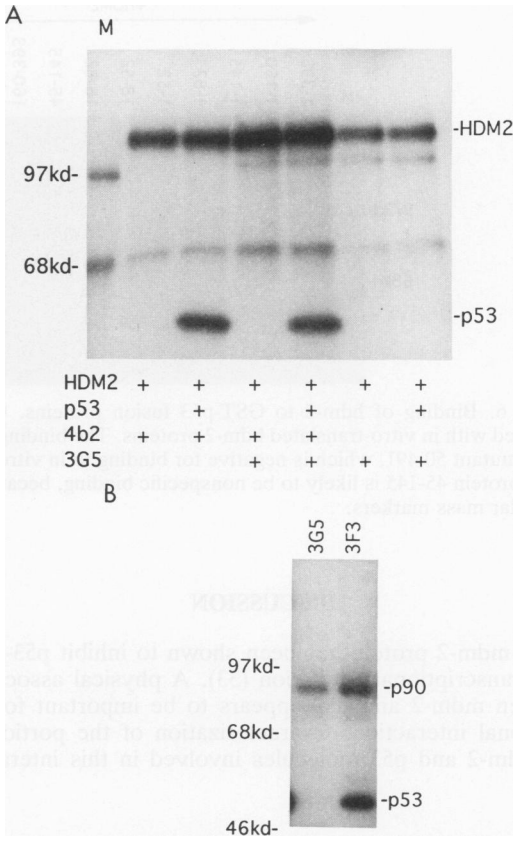

FIG. 5. (A) An antibody with epitopes between residues 59 and 89 could not precipitate the hdm-2–p53 complex. hdm-2 programmed lysate or the preincubated mixture of hdm-2 and p53 lysates was precipitated with 4B2 and 3G5 antibodies. 4B2 but not 3G5 coprecipitated p53 when it was complexed with hdm-2. The presence of hdm-2–p53 complexes in the 3G5 reaction can be demonstrated by including 4B2 in the immunoprecipitation. (B) Immunoprecipitation of A1 cell lysates by using 3G5 and 3F3. A1 cells were metabolically labeled with [^{35}S]methionine. The cellular proteins were immunoprecipitated with the 3G5 and 3F3 antibodies. 3F3 precipitates both p90 (rat mdm-2) and p53; 3G5 only precipitates p90. M (A and B), molecular mass markers.

hdm-2 binding. Glutathione-Sepharose beads were incubated with *E. coli* lysates expressing the GST fusion proteins. hdm-2 was translated and labeled with [^{35}S]methionine in rabbit reticulocyte lysates as described previously. The in vitro translation mixture was then incubated with the glutathione beads loaded with GST-p53 fusion proteins. The beads were washed, and the bound labeled hdm-2 protein was detected by boiling followed by SDS-PAGE. hdm-2 deletion mutants defective for p53 binding in coimmunoprecipitation assays (Fig. 3) were employed as negative controls for these experiments. The results, shown in Fig. 6 and summarized in Fig. 7, demonstrated that the first 52 amino acid residues of the p53 protein are sufficient for this interaction with hdm-2 protein in vitro. Other portions of the p53 protein, when fused to GST, either did not bind to hdm-2 protein or showed only weak and nonspecific binding. The hdm-2 mutants defective for p53 binding did not bind significantly to p53-GST fusions. Therefore, hdm-2 binds to the N-terminal domain of the p53 protein, which contains the acidic residues shown previously to transactivate a test gene and to promote transcription (45).

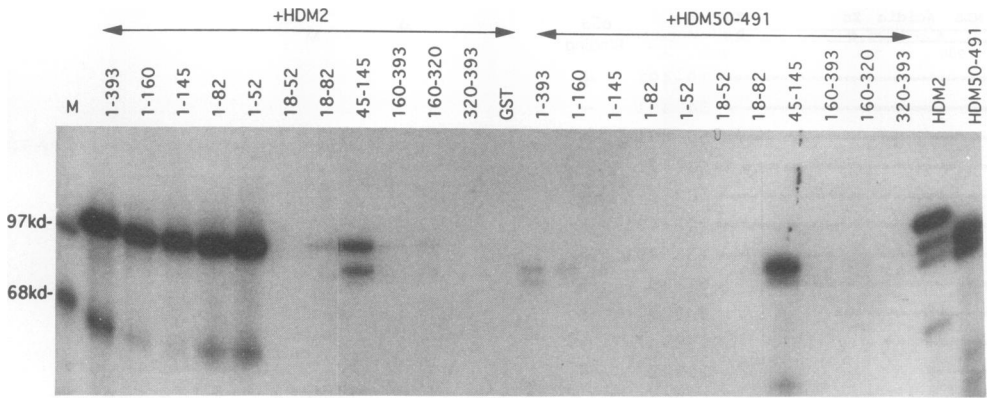

FIG. 6. Binding of hdm-2 to GST-p53 fusion proteins. Glutathione-Sepharose 4B beads loaded with GST-p53 fusion proteins were incubated with in vitro-translated hdm-2 proteins. The binding of hdm-2 proteins to the beads was detected by SDS-PAGE and fluorography. hdm-2 mutant 50-491, which is negative for binding to in vitro-translated p53 was used as a control. The weak binding of hdm-2 to GST-p53 fusion protein 45-145 is likely to be nonspecific binding, because this mutant also binds to the hdm-2 deletion mutant at a similar affinity. M, molecular mass markers.

DISCUSSION

The mdm-2 protein has been shown to inhibit p53-mediated transcriptional activation (33). A physical association between mdm-2 and p53 appears to be important for this functional interaction. Characterization of the portions of the mdm-2 and p53 molecules involved in this interaction should therefore provide useful information about the biological consequences of these protein complexes.

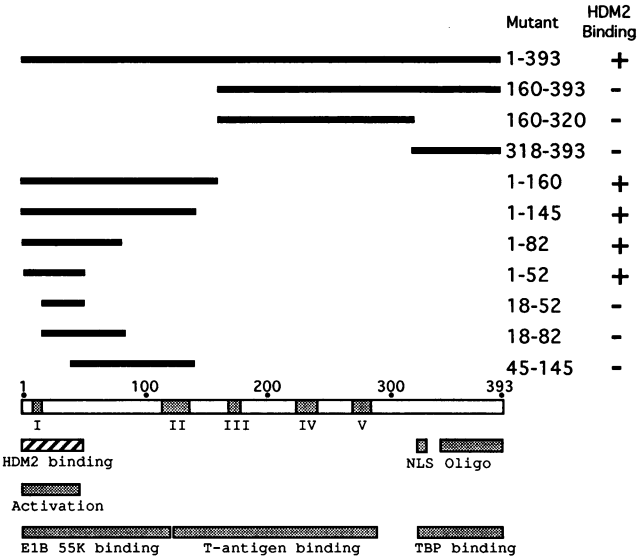

FIG. 7. Summary of hdm-2 binding domain mapping. The open bar represents the p53 polypeptide, and the portions of p53 that are fused to GST are represented by the solid bars, along with their hdm-2 binding activities as assayed in the experiment shown in Fig. 6. The region of p53 that is sufficient for specific binding to hdm-2 is located between residues 1 and 52 (represented by the shaded box below the p53 diagram). Other known p53 functional domains are also shown by shaded boxes. They include the transactivation domain AA1-42 (45), adenovirus E1B 55K binding domain AA1-123 (15), SV40 T-antigen binding region AA123-285 (14, 44), nuclear localization signal (NLS) AA316 (39), oligomerization (Oligo) region AA344-393 (31, 43), the evolutionarily conserved regions I to V (41), and the region for interaction with the TATA-binding protein (TBP) AA320-393 (39a).

This report identifies the regions of the p53 and hdm-2 proteins involved in specific complex formation. Amino acid residues 19 to 102 of hdm-2 and 1 to 52 of p53 appear to interact to form a heterodimer complex. Additional regions of the hdm-2 protein are likely required to stabilize this interaction. This N-terminal region of hdm-2 shares no significant homology to the viral oncogene products that bind to p53 protein or to known functional motifs. The region between residues 19 and 102 of hdm-2 is highly conserved between humans and mice, having 93% identity compared with an average of 82% identity for the entire protein, suggesting that this region is critical for the function or regulation of hdm-2. The role of this region in complexing with p53 is further supported by the observation that monoclonal antibody 3G5, with an epitope located within this region, failed to immunoprecipitate hdm-2 protein in complex with p53. The inability of this antibody to dissociate preformed hdm-2–p53 complexes suggests that either its affinity to hdm-2 is significantly lower than p53, or it recognizes a subset of hdm-2 proteins which do not bind to p53. This could be due to a conformational change or even a different posttranslational modification in this region of the protein. This is consistent with the results of the deletion mapping of the mdm-2–p53 association site.

The amino acid sequences of hdm-2 and mdm-2 suggest that they contain putative metal binding domains (zinc fingers) characteristic of many DNA binding proteins (11, 20), and highly acidic regions similar to that of transcriptional activators (32). It is intriguing that the p53 binding domain in hdm-2 is distinct from the regions thought to be essential for transcriptional function. The organization of the domains of hdm-2 would suggest that it is a transcription factor regulated by or regulating the p53 protein. It is also possible that hdm-2 uses p53 as a functional partner. The deletion mutants generated in this study should be valuable for further analysis of the function of hdm-2 and the role of its interaction with p53.

It has been demonstrated that overexpression of mdm-2 can abrogate the transcriptional activation by p53. By complexing with p53, mdm-2 may do so in two ways; it may alter or inhibit the DNA binding activity of p53, thereby inhibiting

its transactivation function. It is known that direct sequence-specific DNA binding near promoters is critical for p53-mediated transactivation (7, 17, 19, 50). Alternatively, mdm-2 may complex with p53 and interfere with its ability to interact with other proteins that mediate transcriptional activation. The mapping of the hdm-2 binding domain to the N-terminal acidic transcription activation region of p53 suggests that it is possible that the binding of hdm-2 to p53 would interfere with its ability to interact with the proteins that constitute the transcription machinery on the DNA.

The frequent mutation of p53 in human neoplasms suggests that the activities of wild-type p53 are inhibitory to malignant growth. Inactivation of p53 either by mutation or binding to other proteins is a common step in the development of many human tumors. p53 is the target of oncogenes encoded by DNA tumor viruses (reviewed by Levine [24]). The SV40 T antigen binds to the evolutionarily conserved central region of p53 (14, 44) and inhibits its sequence-specific DNA binding, resulting in the loss of transcription activation (1, 30) (Fig. 6). Human papillomavirus E6 proteins bound to p53 and caused its degradation through the ubiquitin-dependent pathway (38). The adenovirus E1B 55-kDa protein binds to the N-terminal region of p53 and inhibits transactivation (48). hdm-2 may modulate p53 function by binding to its transactivation domain. Therefore, it appears that cells do use mechanisms similar to those employed by the tumor viruses to regulate p53 function.

The presence of multiple structural motifs in the mdm-2 protein leaves open the possibility that mdm-2 may be more than just a regulator of p53. It is possible that mdm-2 itself can function as a transcription factor, controlling a set of target genes. Its p53 binding domain also provides the ability to regulate p53 function, thereby extending its effect to the set of p53 target genes that may be involved in growth arrest and differentiation. These ideas suggest that p53 and mdm-2 are likely to regulate each other and that they form a key element in a signal pathway for growth regulation. This is indeed the case. Recent experiments have shown that the *mdm-2* gene contains a p53 responsive element which can be positively regulated by wild-type p53 (47). Thus, the expression of mdm-2 is normally controlled by the levels of p53 protein, and when overexpression of mdm-2 inhibits the activation function of p53, it results in the downregulation of the *mdm-2* gene itself. In this way, the levels of mdm-2 are autoregulated.

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