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# Hot-Spot p53 Mutants Interact Specifically with Two Cellular Proteins during Progression of the Cell Cycle

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**Inactivation of both alleles of the p53 gene is commonly found in human cancers. In contrast to mutations of the retinoblastoma gene, certain altered forms of p53 gain growth-promoting functions. To explore the mechanisms underlying this gain of function, we have identified two nuclear proteins, with molecular masses of 42 and 38 kDa, respectively, that are specifically associated with p53 mutated within the simian virus 40 T-antigen-binding domain, "hot spots" found in many human tumors. These mutants transactivate the multiple-drug resistance gene promoter and cause cells to grow to higher density. Both the mutated p53 complex with p42 and p38 increase when cells enter S phase of the cell cycle but decrease in $G_1$ and M phases, suggesting that they may have a role in promoting cell growth.**

The p53 protein was first identified by its affinity for the simian virus 40 (SV40) large T antigen and coimmunoprecipitation from extracts of SV40-transformed cells by using an anti-SV40 tumor serum (35, 37). It was originally classified as a tumor antigen or an oncoprotein, because the cloned p53 could transform primary rat cells after cotransfection with an activated *ras* oncogene (16, 29, 44). The finding that p53 is frequently mutated in human cancers (27, 42), however, resembled similar observations that documented mutations of the retinoblastoma gene (3). This led to the notion that the mutated but not the wild-type form of p53 can transform cells. Indeed, the cloned p53 cDNA with cotransforming activity contained mutations (19). Conversely, introduction of wild-type p53 suppressed transformation in primary rat embryonic cell assay (19), and in human tumor cells, expression of wild-type p53 can suppress neoplastic phenotypes, including tumorigenicity in nude mice (1, 4, 6, 65). These results suggest that normal p53 functions as a tumor suppressor.

The p53 gene is the most commonly altered locus in human tumors (27, 36). Typically, one allele of p53 in these tumors is not expressed because of a rearrangement or deletion, and the remaining allele sustains a missense mutation. If cells contain both mutant and normal alleles of p53, they display a wild-type phenotype, suggesting that normal p53 is dominant to the mutant form (4). This finding is consistent with the observation that phenotypically normal fibroblasts could be obtained from patients with the Li-Fraumeni syndrome (39, 59), indicating that inactivation of the remaining wild-type p53 allele is essential for the mutant p53 to participate in oncogenesis.

A broad spectrum of mutations of p53 has been found in human tumors (27, 36). The correlation of more advanced stages of cancer with mutations of p53 has corroborated the suggestion that mutant p53 may play an important role in cancer progression (49). Certain mutated forms of p53 not only lose growth suppression but also gain a growth-promoting phenotype (11, 46). Frequently occurring mutations ("hot spots") are found at codons 175, 248, 249, and 273 (27, 36, 46) of p53 molecules that have invariably lost their ability to bind to SV40 large T antigen (46). However, not all missense mutations of p53 found in human cancers result in the same phenotype (26, 64, 70). These observations suggest that the different mutations of p53 found in tumor samples may not contribute equally to tumorigenesis.

Numerous studies have examined the relationship between p53 structure and its potential biological activities (24, 58). On the basis of these experiments, p53 protein has been divided into three regions: the amino terminus, containing the transcriptional activation region (18, 43, 48); the central portion of the molecule, containing sequences specific for DNA-binding activity (8a, 44a, 67) in which the majority of oncogenic mutations are located (27, 36, 42, 61); and the carboxyl terminus, containing the oligomerization domain (60) and nuclear localization sequences (57). Recent results strongly suggest that wild-type p53 protein is a transcription factor which can transactivate or repress specific promoters (15, 17, 21, 33). Wild-type p53 protein interacts with several oncoproteins encoded by DNA tumor viruses, including SV40 large T antigen, E1B of adenovirus, and E6 of human papillomavirus (35, 37, 52, 68). Interestingly, the domains of p53 that bind to these oncoproteins have been mapped to different regions. The central part of p53 contains the SV40 T-antigen-binding (T-binding) domain (30, 51, 62). Many missense p53 mutants fail to bind to T antigen and also lose transactivation ability (47, 64), suggesting that a specific conformation of p53 may be required to maintain normal function (23).

We have observed that cells containing p53 mutated at codon 273 grow more aggressively than cells harboring p53 null alleles (4). This and other previous observations indicated that certain mutant forms of p53 may have gained growth-promoting activity (11, 46). To explore the mechanisms by which these altered species of p53 promote cell growth, we have sought to determine whether any cellular proteins interact specifically with mutant p53. We initially observed that the p53 encoded by a gene mutated at codon 273 associated with two nuclear proteins, p42 and p38. Further investigations have demonstrated that p53 with missense mutations in the T-binding domain binds to these two nuclear proteins, whereas the wild-type or other mutants do not. We then constructed five p53 mutants, three with missense mutations within the T-binding domains and two outside the T-binding domains. p53 with the mutation within the SV40 T-binding domains strongly

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transactivated the multiple-drug resistance gene 1 (*MDR1*) promoter and allowed cells to grow to a higher density. The specific complex formed between the mutated p53 protein and the two nuclear proteins increased in abundance during S phase of the cell cycle. This observation is compatible with the gain-of-function phenotype exhibited by certain mutated forms of p53.

## MATERIALS AND METHODS

**Cell lines and cell culture.** Cell lines HOS-SL, T47D, HT-3, Saos-2, SW837, SW620, HT-29, U-2OS, MOLT-4, MB-468, and A673 were obtained from American Type Cell Collection. All cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (FCS), except MOLT-4 cells, which were grown in RPMI 1640 with 10% FCS.

**Immunoprecipitation.** Immunoprecipitation was performed as previously published (4). Briefly, about $10^7$ cells from each cell line were metabolically labeled with [$^{35}$S]methionine for 2 h and subsequently lysed in ice-cold lysis buffer (50 mM Tris-HCl [pH 7.4], 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride). After incubation of the clarified lysate with anti-p53 antibody at 4°C for 2 h, protein A-Sepharose beads were added, and the mixture was incubated for another 30 min at 4°C. The beads were washed extensively with lysis buffer and boiled in sodium dodecyl sulfate (SDS) sample buffer, and then the immunoprecipitates were separated by SDS–11.5% polyacrylamide gel electrophoresis (PAGE).

**V8 protease digestion.** The experimental procedure was adapted from that of Cleveland et al. (9). Briefly, immunoprecipitates were first separated by electrophoresis. The resultant gel was dried without any further treatment and exposed to X-ray film for 9 h at room temperature. The bands corresponding to p53 (HT-29), p42, and p38 (HT-29, SW620, and SW837) were cut out with a razor blade and then equilibrated in 0.125 M Tri-HCl (pH 6.8)–0.1% SDS buffer for 30 min at room temperature. After soaking, the gel slices were loaded into an SDS–17.5% polyacrylamide gel with a 5-cm stacking gel. The gel slices were overlaid with equilibrated buffer containing 20% glycerol, a known amount of *Staphylococcus* V8 protease in 10% glycerol, 0.001% bromophenol blue, 0.125 M Tri-HCl (pH 6.8), and 0.1% SDS buffer. The gel was run at 25 mA until the tracking dye reached the top of the resolving gel and then run at 40 mA until the tracking dye reached the bottom of the gel. The gel was processed and then dried for autoradiography.

**Plasmid construction and preparation of retroviruses.** Retroviral constructs carrying mutant p53E and wild-type p53B have been previously described (4). cDNA encoding mutant p53I was prepared by the reverse transcriptase-PCR method, using BT549 mRNA, which contains a missense mutation (Arg-249→Ser) of p53. cDNAs encoding p53K, p53H, and p53N were generated by site-specific mutagenesis using PCR methods. The six p53 cDNAs were then inserted individually into the expression vector CEP4, which contains a cytomegalovirus promoter (Invitrogen, San Diego, Calif.), for transient transfection assays. Two additional retroviruses carrying p53K and p53I were prepared by the protocol described elsewhere (4). All of the mutant p53 constructs were confirmed by sequence analysis using the dideoxy-chain termination method.

**Preparation of antiserum against p42 and p38.** p42 and p38 proteins were purified from $10^{10}$ SW620 cells by affinity chromatography, using the p53 antibody pAb122 bound to protein G-Sepharose beads. Immunocomplexes were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (Immobilon-P; Millipore) membrane. After brief Coomassie blue staining (40% methanol, 5% acetic acid, 0.02% Coomassie blue), the bands corresponding to p42 and p38 were cut out with a razor blade. Approximately 70 μg of p42 and 50 μg of p38 were obtained. Membrane slices containing p42 and p38 were then inserted subcutaneously into a mouse for generation of antiserum.

**Transfection and CAT assay.** Transfections were carried out with CV-1 cells by the calcium phosphate-DNA coprecipitation method (55). Ten micrograms of each plasmid containing mutant p53 was cotransfected with 5 μg of the plasmid containing the *MDR1* promoter driving the chloromphenicol acetyltransferase (CAT) gene (8). CAT activities were measured 40 h after transfection, using equal amounts of total cellular proteins.

**Cell cycle synchronization.** HT-29 cells were synchronized by the double-thymidine block method (10). Briefly, thymidine was added, to a final concentration of 2 mM, to medium containing 10% FCS. After 14 h, cells were washed three times with phosphate-buffered saline (PBS) and then refed with fresh medium containing 10% FCS. The same amount of thymidine was added again after 10 h and then removed from the medium after 14 h of incubation. Cells were washed three times in PBS, and fresh medium without thymidine was added to the cells. Aliquots of cells were then taken at different time points for metabolic labeling with [$^{35}$S]methionine (100 μCi/ml). Cells were labeled for 2 h and used for immunoprecipitation by pAb122. Aliquots of cells from the same time points were checked for S-phase DNA synthesis in a [$^3$H]thymidine uptake assay and for entry into M phase by calculation of mitotic index. For the [$^3$H]thymidine uptake experiment, 1 ml of warm Dulbecco modified Eagle medium plus 1 μCi of [$^3$H]thymidine per ml was added to cells for 30 min. The medium was then removed, and 1 ml of cold 5% trichloroacetic acid was added. The cells were incubated for 10 min and then gently washed three times with cold Hanks balanced salt solutions (Gibco/BRL). Cells were lysed with 1 ml of 0.1% SDS–0.01 N NaOH solution. Two hundred microliters of lysate was counted by liquid scintillation. Mitotic index was measured by simply examining the cells under a microscope; the mitotic cells were counted and scored as a percentage of cells in the entire area examined.

## RESULTS

**P38 and p42 coimmunoprecipitated with a mutant p53 (Arg-273 to His) but not with wild-type p53.** We have introduced, via a retrovirus-mediated gene transfer method, either the wild-type p53 gene (p53BH) or codon 273 mutant p53 (p53EN) into A673 human peripheral neuroepithelioma (PNET) cells, which contain no detectable p53 protein. PNET cells that ectopically expressed the wild-type p53 lost their tumorigenic ability, while those expressing the mutant p53 retained tumorigenicity (6). Compared with the parental cells, the cells expressing the mutant p53 grew to a much higher saturation density, suggesting that the mutant form of p53 may have gained a growth-promoting activity. This growth characteristic was also seen when a human osteosarcoma cell line, Saos-2, which also lacks p53 (40), was used in a similar experiment (4). To explain the possible mechanism of this phenomenon, we hypothesize that the growth-promoting forms of p53 may associate with specific cellular proteins. To identify these potential cellular proteins, PNET cells were metabolically labeled with [$^{35}$S]methionine and immunoprecipitated with a monoclonal antibody, pAb122, which specifically recognizes p53 (23). As shown in Fig. 1, two proteins with

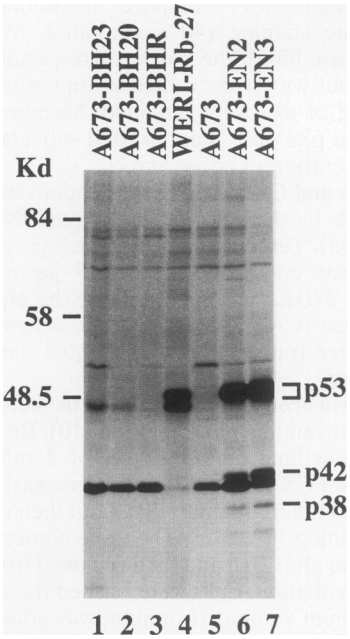


FIG. 1. p38 and p42 coimmunoprecipitated with p53 mutated at codon 273 but not with wild-type p53. A673 human PNET cells were infected with retrovirus Vp53BH, which carries a wild-type p53, or Vp53EN, which has the p53 mutated at codon 273. Cells from individual clones were metabolically labeled with [$^{35}$S]methionine, and their lysates were immunoprecipitated with an anti-p53 antibody, pAb421. Cell clones A673-BH23 (lane 1) and A673-BH20 (lane 2) contain wild-type p53 protein, A673-BHR (lane 3) and A673 (lane 5) contain no p53 protein, and A673-EN2 (lane 6) and A673-EN3 (lane 7) contain mutant p53. WERI-Rb-27 (lane 4) was included as a position marker for p53 protein. p42 and p38 coimmunoprecipitated only from A673-EN2 and A673-EN3 (lanes 6 and 7).

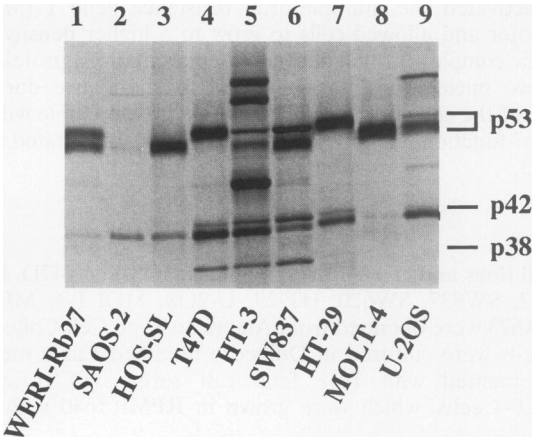


FIG. 2. Coimmunoprecipitation of p38 and p42 with p53 mutants in different human tumor cell lines. All cells were treated as described for Fig. 1. p42 and p38 were coimmunoprecipitated with mutant p53 from cells lines of T47D, HT-3, SW837, and HT-29 but not from the other cell lines. In HT-3 cells, multiple bands of p53 protein, varying in size, were reproducibly observed. The precise reason for this pattern is not known.

molecular masses of 38 and 42 kDa were specifically coimmunoprecipitated with the mutated form of p53 (lane 6 and 7). These two proteins were not detected in the immunocomplex from either parental A673 cells (lane 5) or A673-BH cells (lanes 1 and 2), which both express wild-type p53 protein, or A673-BHR (lane 3), which is a revertant of A673-BH that has lost expression of wild-type p53. Similar results were obtained with Saos-2 cells. When either a wild-type or mutant p53 gene was introduced into Saos-2 cells, p42 and p38 were specifically coimmunoprecipitated with the mutated form of p53 (data not shown).

**p53 with mutations within the T-binding domain associates with p42 and p38 in different human tumor cells.** To test the possibility that only certain mutant forms of p53 can complex with p38 and p42, several tumor cell lines that contain different mutations of p53 were examined (Table 1). The cell lines from osteosarcoma (U-2OS), leukemia (MOLT-4) (7), and retinoblastoma (WERI-Rb-27) contain p53 protein that binds to SV40 large T antigen and is assumed to be wild type, at least within the T-binding domains. The other tumor cell lines contain p53 mutated at the following codons: Arg-156→Pro (HOS-SL) (50), Leu-194→Phe (T47D) (42), Gly-245→Val (HT-3) (53), Arg-248→Trp (SW837) (42, 49), and Arg-273→His (HT-29) (49). The p53 mutations in T47D, HT-3, SW837, and HT-29 occur either within the first (T47D) or the second (HT-3, SW837, and HT-29) T-binding domain. As shown in Fig. 2, p42 and p38 were coimmunoprecipitated from cell lines T47D (lane 4), HT-3 (lane 5), SW837 (lane 6), and HT-29 (lane 7) but not from cell lines WERI-Rb-27 (lane 1), Saos-2 (lane 2), HOS-SL (lane 3), MOLT-4 (lane 8), and

TABLE 1. Summary of p42 and p38 coimmunoprecipitation with mutated p53 protein in different cell lines

| Cell line | Tumor type | Mutation | T binding | Reference | p42/p38 binding |
|---|---|---|---|---|---|
| WERI-Rb-27 | Retinoblastoma | | + | 5a | − |
| Saos-2 | Osteosarcoma | Deletion | − | 40 | − |
| HOS-SL | Osteosarcoma | Arg-156→Pro | + | 50 | − |
| T47D | Breast carcinoma | Leu-194→Phe | − | 42 | + |
| HT-3 | Cervical carcinoma | Gly-245→Val | − | 53 | + |
| SW837 | Colon carcinoma | Arg-248→Trp | − | 42 | + |
| BT549 | Breast carcinoma | Arg-249→Ser | − | 2a | + |
| SW620 | Colon carcinoma | Arg-273→His | − | 49 | + |
| MB-468 | Breast carcinoma | Arg-273→His | − | 42 | + |
| HT-29 | Colon carcinoma | Arg-273→His | − | 49 | + |
| A673-16E | PNET | Arg-273→His | − | 6 | + |
| MOLT-4 | Leukemia | − | + | 7 | − |
| U-2OS | Osteosarcoma | − | + | 10a | − |

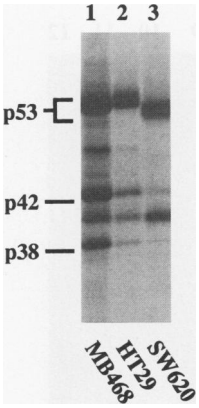


FIG. 3. The same p53 mutant (Arg-273→His) in different cell backgrounds is associated with p42 and p38. [$^{35}$S]methionine-labeled cell lysates from MB-468 (lane 1), HT-29 (lane 2), and SW620 (lane 3) were immunoprecipitated with anti-p53 antibody pAb122, and p42 and p38 were also detected as indicated.

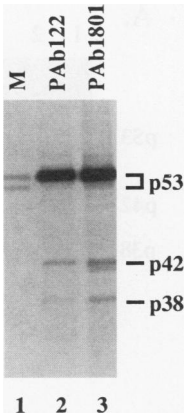


FIG. 4. p42 and p38 were coimmunoprecipitated by monoclonal antibodies that recognized different epitopes of p53 protein. MB-468 cells were labeled with [$^{35}$S]methionine, and the clarified lysate was immunoprecipitated with either pAb1801, which recognizes the N-terminal portion of p53 protein (lane 2), or pAb122, which recognizes the C-terminal portion of p53 protein (lane 3). The p53 from WERI-Rb-27 served as a position marker for p53 (lane 1).

U-2OS (lane 9). Since the most frequently mutated hot-spot region of p53 in human tumors is located precisely within the T-binding domains, these results indicate that the hot-spot p53 mutants which are mutated within the T-binding domains encode protein that specifically associated with the two cellular proteins, p42 and p38.

To demonstrate the lack of cell type specificity in the association of p38 and p42 with the mutated forms of p53, we examined one of the most frequently occurring p53 mutations in human tumors, Arg-273→His, in three different tumor cell lines, HT-29 (colon carcinoma), SW620 (colon carcinoma) (49), and MB-468 (breast carcinoma) (42). As shown in Fig. 3, p42 and p38 coimmunoprecipitated with p53 in cell line MB-468 (lane 1), HT-29 (lane 2), or SW620 (lane 3). These results suggest that complex of p53, p42, and p38 may form regardless of cell type.

**p42 and p38 are distinct from p53.** To demonstrate that the coimmunoprecipitation of p42 and p38 with p53 is not due to antibody cross-reaction, we used two different monoclonal anti-p53 antibodies: pAb122, which recognizes the C-terminal portion of p53 protein, and pAb1801, which recognizes the N-terminal portion of p53 protein, in the following experiment. MB-468 cells were metabolically labeled with [$^{35}$S]methionine, and equal amounts of the cell lysate were immunoprecipitated with either pAb122 or pAb1801. As shown in Fig. 4, the immunocomplexes brought down by pAb122 (lane 2) and pAb1801 (lane 3) both contained p42 and p38 proteins. These results indicated that the coimmunoprecipitation of p42 and p38 with p53 is unlikely to be due to a spurious antibody cross-reaction.

Second, to demonstrate that the same p42 and p38 proteins were coprecipitated from the different tumor cell lines and that these two proteins are different from each other and from p53, we selected three tumor cell lines, HT-29, SW620, and SW837, to isolate p42 and p38 proteins for V8 protease peptide mapping (Fig. 5). The cells were labeled with [$^{35}$S]methionine and then immunoprecipitated with monoclonal antibody pAb421. After separation by SDS-PAGE, the bands corresponding to p53 from HT-29 and p42 and p38 from HT-29, SW620, and SW837 were isolated and digested with V8 protease. The peptide patterns of p53, p42, and p38 are clearly different, which suggests that these three proteins are distinct and that p42 and p38 are not subfragments of p53. Moreover, the V8 protease digestion patterns of p42 from HT-29 (lanes 4 to 6), SW620 (lanes 7 to 9), and SW837 (lanes 10 to 12) were identical, indicating that the p42 coprecipitated by the same mutated forms of p53 in different cell lines (HT-29 and SW620) or by different mutant forms of p53 (SW837 and HT-29) is the same protein. Similar results were also obtained for p38 (data not shown).

**Antibodies against p42 and p38 coimmunoprecipitate mutant p53.** To unequivocally demonstrate that p42 and p38 specifically interact with mutant p53 requires a reciprocal immunoprecipitation experiment. We purified 70 μg of p42 and 50 μg of p38 to serve as antigens for immunization of a mouse. As shown in Fig. 6A, the mouse immune serum specifically recognized p42 and p38 when different amounts of SW837 cell lysate were analyzed by direct Western blot (immunoblot) analysis. These results reinforced the conclusion that p42 and p38 are not structurally related to p53 since p53 was not detected by the antiserum. However, the mutant p53 of HT-29 tumor cells was coimmunoprecipitated by the p38/p42 antiserum (Fig. 6B). These results confirm that p42 and p38 form specific complexes with p53 mutated within T-binding domains.

**Hot-spot p53 mutants transactivate the *MDR1* promoter and allow cells to grow to higher density.** To directly compare the biochemical properties of different p53 mutants in the identical cell background, five missense mutants, including mutations at codons 132, 156, 175, 249, and 273, were constructed. As shown in Fig. 7, mutations of codons 175, 249, and 273 are located at the hot-spot region and within the T-binding domains, whereas mutations 132 and 156 are not. Expression of each of these mutant p53 alleles by the cytomegalovirus promoter of the CEP-4 vector was verified by transient transfection of Saos-2 cells followed by immunoprecipitation and Western blotting (data not shown).

Normal monkey kidney cells (CV-1) were cotransfected with the p53 mutant constructs and the *MDR1* promoter driving the CAT reporter gene. As shown in Fig. 8, mutants with mutations at codons 175, 249, and 273 transactivated the *MDR1* promoter by about threefold. In contrast, mutants with mutations at codons 132 and 156 repress the *MDR1* promoter activity by about three- to fourfold. Thus, the difference in

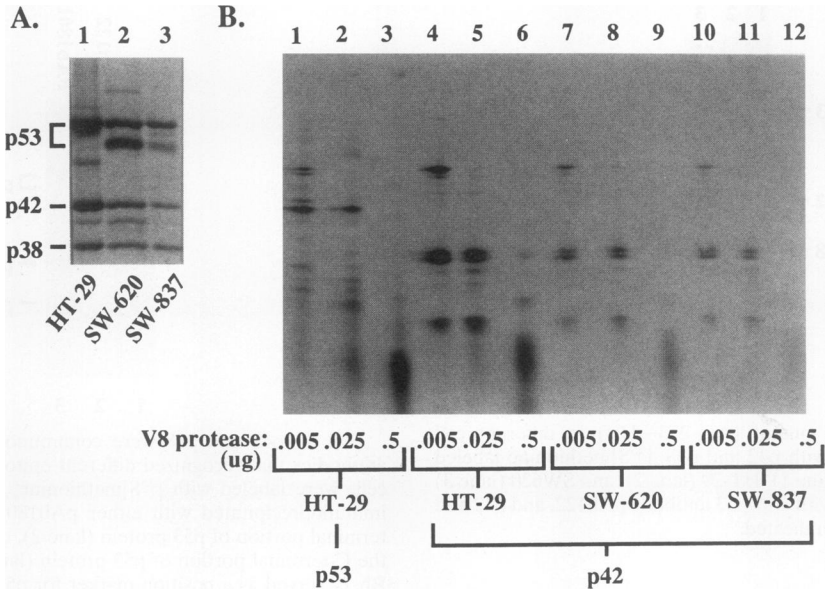


FIG. 5. p42 is structurally different from p53 by V8 protease peptide mapping. (A) Three cell lines, HT-29 (lane 1), SW620 (lane 2), and SW837 (lane 3), were labeled with [$^{35}$S]methionine, and the lysates were immunoprecipitated with pAb421. All three different p53 mutants from these cells were coimmunoprecipitated with p42 and p38. (B) The bands corresponding to p53, p42, and p38 were isolated and subjected to in situ V8 protease digestion. The fragments generated by digestion of p53 from HT-29 (lanes 1 to 3) and by digestion of p42 from HT-29 (lanes 4 to 6), from SW620 (lanes 7 to 9), and from SW837 (lanes 10 to 12) are shown. The amounts of V8 protease used for digestion are indicated.

transactivation of the *MDR1* promoter between these two groups of p53 mutants is about 10-fold. These results suggest that the hot-spot p53 mutants may enhance transactivation of the *MDR1* gene, consistent with the published results (8, 11).

One of the features of mutant p53 found in many tumors is enhancing cell growth. Certain p53 mutants can promote cells to grow to higher saturation density compared with cells that do not express p53 (null mutants) (4). To examine the effects of the p53 mutants constructed in this study on cell growth, individual cell lines expressing each of these mutant p53 proteins were established by using retrovirus-mediated gene transfer, which favors a single-copy gene integration. Individual Saos-2 cell lines were obtained by infection with a retrovirus carrying p53 with mutations at codons 132, 249, and 273. Multiple clones were isolated, and two clones for each mutant were randomly chosen for growth saturation density assay. The parental Saos-2 cells and clones expressing the codon 132 mutant grow to similar densities, whereas clones expressing codon 249 and 273 mutants reach a four- to fivefold-higher cell density (Fig. 9). These results are compatible with those showing differential transactivation of the *MDR1* promoter and suggest that the hot-spot p53 mutants have indeed gained growth-promoting activity.

**p42 and p38 associate with mutant p53 in the nucleus.** Since p53 is a nuclear protein, interaction of mutant p53 with p38 and p42 would be expected to occur in the nucleus. To examine the subcellular location of these complexes, SW837 cells were metabolically labeled with [$^{35}$S]methionine and fractionated into membrane, cytoplasmic, and nuclear fractions. Aliquots of each fraction were immunoprecipitated with pAb122 or precipitated by glutathione-Sepharose beads that absorbed glutathione *S*-transferase (GST) as a cytoplasmic marker (63). As shown in Fig. 10, p42, p38, and mutant p53 were detected in the total cell lysate (lane 1) and nuclear fraction (lane 2) precipitated by pAb122 but not in the cytoplasmic (lane 3) or membrane (lane 4) fraction. In contrast, the 23 kDa GST was

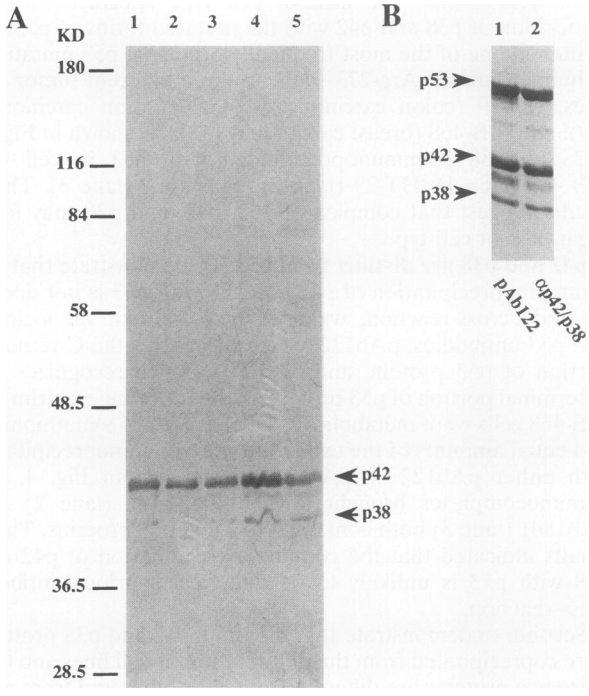


FIG. 6. Antiserum prepared against p42 and p38 coprecipitates mutant p53. (A) Specificity of mouse antiserum against p42 and p38 proteins. Different amounts of SW837 cell lysates, $10^5$ (lane 2), $2 \times 10^5$ (lane 3), $5 \times 10^5$ (lane 4), and $10^6$ (lane 5) cells, were used for Western blotting analysis with p42/p38 antibodies as probes. Original antigens purified by immunoprecipitation with pAb122 served as a positive control (lane 1). The arrows indicate migration of p42 and p38. (B) HT-29 cells were labeled with [$^{35}$S]methionine and immunoprecipitated with either pAb122 or anti-p42/p38 antibody. Complexes containing p42, p38, and mutant p53 were immunoprecipitated by pAb122 (lane 1) and also by anti-p42/p38 (αp42/p38; lane 2).

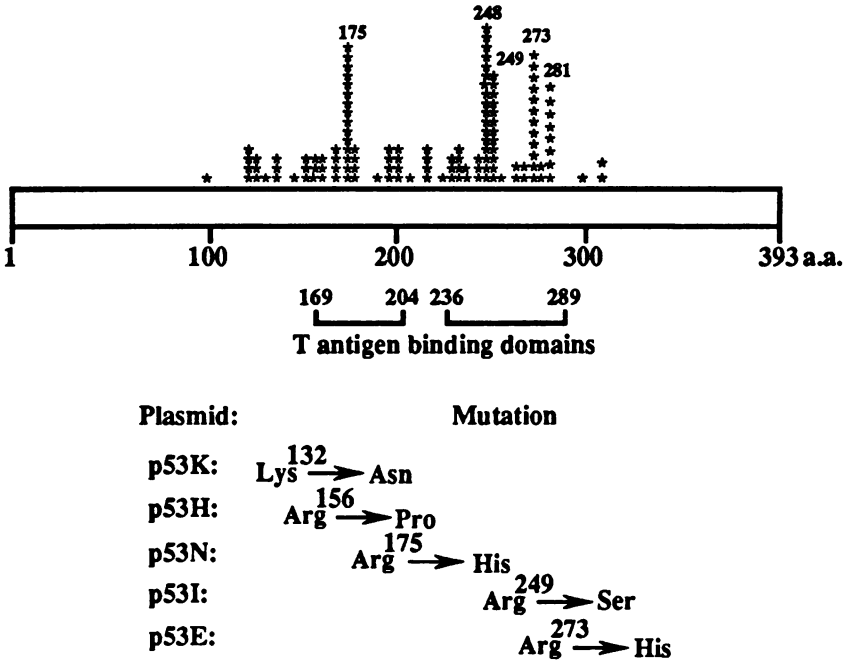


FIG. 7. Diagram of the frequency of p53 mutations found in tumor cells and the corresponding missense mutations of p53 constructs used in this study. Frequency (*) and location of mutations of the p53 gene were obtained from published data (27, 36). a.a., amino acids.

detected in the cytoplasmic fraction (lane 6) but not in the nuclear (lane 5) or membrane (lane 7) fraction. These results demonstrate that p42 and p38 form a complex with mutant p53 in the nucleus.

**The abundance of complex of p42 and p38 with mutant p53 increases during the S phase of the cell cycle.** As described above, p53 mutants that enhance cell growth may be a consequence of complex formation with p42 and p38. We have examined the abundance of this complex at different stages of the cell cycle. HT-29 cells were arrested at mid-$G_1$ phase with a double-thymidine block and then synchronously released to progress through the next cell cycle (10). As previously shown, the abundance of mutant p53 is relatively constant during cell cycle progression. However, the amount of p42 and p38 increased during DNA synthesis (S phase) and decreased when cells are enriched with mitotic figures (M phase) (Fig. 11A and B). Assuming that the amount of p53 is constant during this period, therefore, the amount of p42 and p38 available to complex with p53 was highest during S phase and lower during $G_1$ and M phases (Fig. 11C). Similar results were obtained when a different method was used to synchronize cell cycle progression (data not shown).

## DISCUSSION

Mutations of the p53 gene are widely found in human tumors (27, 36, 46). However, different p53 mutants may not contribute equally to oncogenesis (8, 11, 26, 70). Apparently, only a subset of p53 mutants have gained growth-promoting activity (11). Two such mutants, Arg-175→His and Arg-273→His, transactivated the *MDR1* promoter activity (8, 11) and allowed cells to grow to a higher density (4). The results described here, for several different mutants, confirmed and extended this observation. Furthermore, the same subset of mutated p53 that exhibit growth-promoting activity specifically bind to two nuclear proteins, p42 and p38. The complex

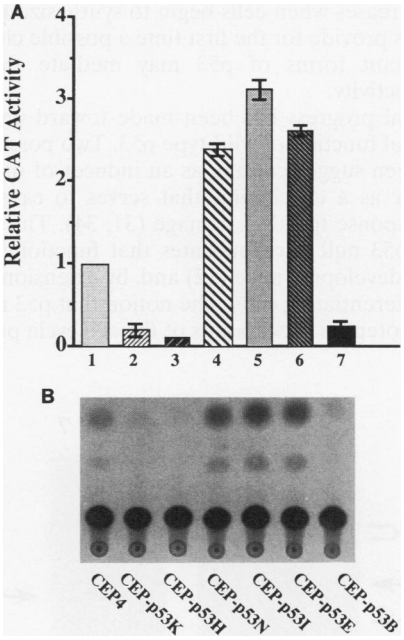


FIG. 8. p53 mutated within the T-binding domain enhances transactivation of the *MDR1* promoter. Six different forms of p53 were subcloned into the CEP4 vector. In each construct, the p53 gene was expressed under regulation of the cytomegalovirus promoter. CV-1 cells were cotransfected with 5 μg of the reporter plasmid DNA (*MDR*-CAT) and 10 μg of various p53 plasmids, using the calcium phosphate-DNA precipitation procedure. CAT activity was determined by using equal amounts of protein lysates. The percentage of acetylated $^{14}$C-labeled chloramphenicol was quantified with a PhosphorImager (Molecular Dynamics) (A). The autoradiograph is shown in panel B. The results were generated from at least three independent transfections. The constructs containing mutant p53 are depicted in Fig. 7.

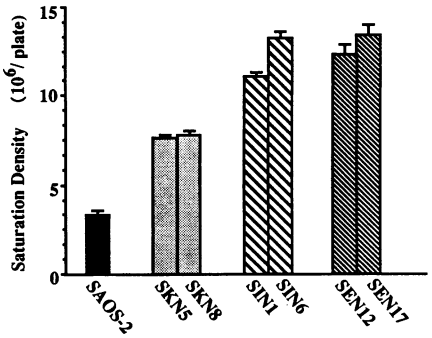


FIG. 9. p53 mutants promote cell growth to higher saturation density. A single copy of the various p53 mutants was introduced into Saos-2 cells by retrovirus-mediated gene transfer. Two separate clones expressing the corresponding mutant protein were used in this assay. About $10^6$ cells were plated in a 60-mm-diameter culture dish, and two dishes from the total of 20 were taken for counting the cell number representing complete saturation. The clones expressing mutant p53 at codon 132 (SKN5 and SKN8) have about twofold-higher saturation density than the parental cells (Saos-2). Cells expressing the other two mutants, at codon 249 (SIN1 and SIN6) and codon 273 (SEN12 and SEN17), have fourfold-higher saturation density than parental Saos-2 cells. Each bar in the histogram represents the mean data from three separate plates of cells.

formed between these proteins may be relevant to enhance progression through S phase, since the abundance of the complex increases when cells begin to synthesize DNA. These observations provide for the first time a possible clue as to how certain mutant forms of p53 may mediate their growth-promoting activity.

Substantial progress has been made toward understanding the biological function of wild-type p53. Two possible roles for p53 have been suggested, one as an inducer of differentiation and another as a checkpoint that serves to cause cell cycle arrest in response to DNA damage (31, 34). The existence of live, adult p53 null mice indicates that functional p53 is not essential in developing mice (12) and, by extension, not critical for most differentiating cells. The notion that p53 may serve as one of the potential checkpoints of the cell cycle progression is

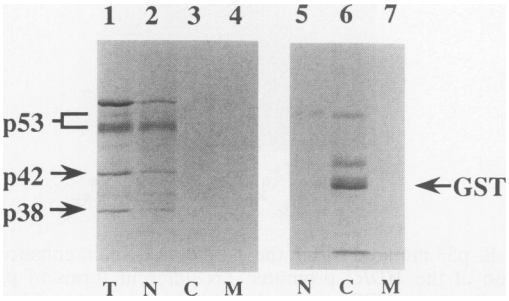


FIG. 10. p42 and p38 are associated with mutant p53 in the nucleus. SW837 cells were metabolically labeled with [$^{35}$S]methionine and fractionated into nuclear (N), cytoplasmic (C), and membrane (M) fractions. Each fraction was immunoprecipitated with pAb122 or with glutathione-Sepharose beads to bring down GST as a cytoplasmic protein marker. The complexes of p53 and p42/p38 were detected in the total cell lysate (lane 1) or nuclear fraction (lane 2) but not in the cytoplasmic (lane 3) or membrane (lane 4) fraction. GST was found in the cytoplasmic fraction (lane 6) but not in either the nuclear (lane 5) or membrane (lane 7) fraction.

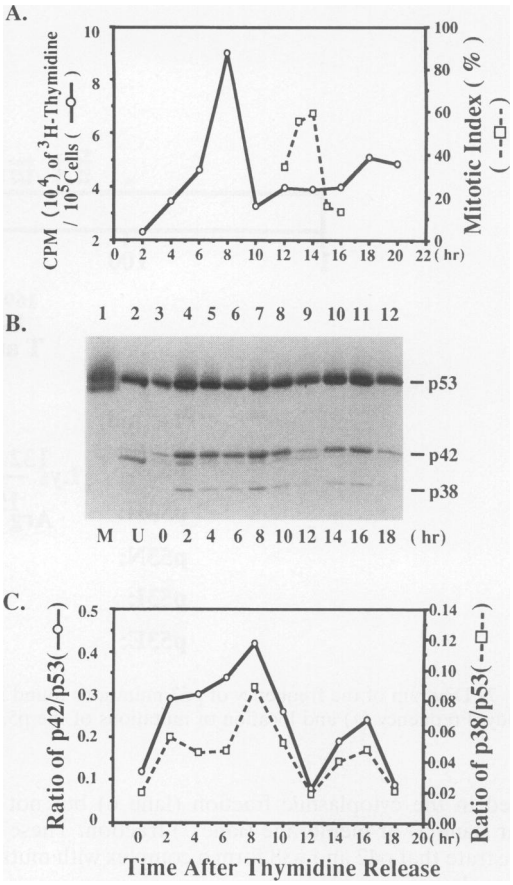


FIG. 11. Association of p53 and p42/p38 during cell cycle progression. HT-29 cells were arrested at early $G_1$, by a double-thymidine block, and then released. At the different time points, aliquots of cells were examined for the status of cell cycle progression by assaying either for [$^3$H]thymidine uptake (DNA synthesis) or for mitotic figures (A). Equal numbers of cells harvested at the corresponding time points were labeled and lysed for immunoprecipitation with antibody pAb122 (B). Unsynchronized cells (U; lane 2) and WERI-Rb-27 cells (M; lane 1) were also included as markers for p53 migration. Quantitation of each p53, p42, and p38 band was performed by densitometry (Molecular Dynamics), and the p42/p53 and p38/p53 ratios were plotted as shown in panel C. The highest p42/p53 and p38/p53 ratios corresponded with the peak of thymidine uptake, which represents S phase.

probably the best-substantiated function of p53 (31, 34). According to this notion, mutations of p53 may inactivate checkpoint functions (13, 31). This rationale was further supported by recent results which demonstrated that mutations of p53 do not augment the frequency of tumor initiation or the rate of tumor promotion but greatly enhance malignant progression (32).

Evidence has accumulated to suggest that the wild-type p53 may serve as a sequence-specific transcription factor (5, 17, 21, 22, 33, 38, 54, 69). Attempting to identify proteins that bind to p53 has been a major focus in the search for more clues to determining the sequence specificity and exact function of p53. At least eight cellular proteins that bind to wild-type p53 have been identified, but most of these are still associated with mutant p53 occasionally (2, 14, 20, 25, 28, 41, 45, 54, 66). To date, there is no cellular protein which has been found to be specifically associated with mutant p53. Previously, a heat shock protein was reported to bind to a mutant p53, Arg-

175→His, which may have a higher cotransforming activity (20). However, other mutants apparently need not be associated with the heat shock protein to gain such strong transforming activity (26). As previously suggested, loss of suppressor activity and acquisition of transforming potential may be separable and may involve at least two distinct functions of p53 (56).

It is interesting that the central region of the wild-type p53 molecule forms a domain which binds to SV40 T antigen (30, 51, 62). Conceivably, certain missense mutations of p53 generate a unique conformation (22) that may facilitate interaction with other cellular proteins either by creating a novel interaction site or by disrupting an interaction with another cellular protein that normally blocks p53-p42-p38 interaction. Our data support this concept. The hot-spot p53 mutants may have a conformation that facilitates formation of complex with other cellular proteins and thereby may confer a growth advantage to transformed cells during tumor progression.

At present we do not know the precise cellular functions of p42 and p38. However, their specific interaction with this gain-of-function subset of p53 mutants argues that the formation of the complex of p53, p42, and p38 may be relevant to growth-promoting activity. It is conceivable that mutant p53 interacts with a group of proteins involved in negative regulation of cell growth. The abundance of stable mutated p53 would be sufficient to sequester such proteins and allow cells to enter S phase. Alternatively, p42 and p38 may form active complexes and turn on genes allowing the entrance into the cell cycle. Identification of the genes encoding p42 and p38 should help clarify the cellular functions of these proteins.

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