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Gas1-Induced Growth Suppression Requires a Transactivation-Independent p53 Function

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In normal cells, induction of quiescence is accompanied by the increased expression of growth arrest-specific genes (*gas*). One of them, *gas1*, is regulated at the transcriptional level and codes for a membrane-associated protein (Gas1) which is down regulated during the G₀-to-S phase transition in serum-stimulated cells. Gas1 is not expressed in growing or transformed cells, and when overexpressed in normal fibroblasts, it blocks the G₀-to-S phase transition. Moreover, Gas1 blocks cell proliferation in several transformed cells with the exception of simian virus 40- or adenovirus-transformed cell lines. In this paper, we demonstrate that overexpression of Gas1 blocks cell proliferation in a p53-dependent manner and that the N-terminal domain-dependent transactivating function of p53 is dispensable for Gas1-induced growth arrest. These data therefore indicate that the other intrinsic transactivation-independent functions of p53, possibly related to regulation of apoptosis, should be involved in mediating Gas1-induced growth arrest.

The nonproliferative state (G₀) in normal cells is characterized by increased expression of a set of genes called *gas* (growth arrest specific) (33). Gas1 is a membrane-associated protein that, when ectopically expressed, blocks the G₀-to-S phase transition of quiescent fibroblasts without interfering with the early serum response (7). Blocking of cell proliferation was also observed in several transformed cells of mouse and human origin but not in cells transformed with simian virus 40 (SV40) or with adenovirus type 5 DNA. Because these viruses are known to inactivate pRb (or pRb-related) and p53 proteins, these data suggested that Rb and/or p53 could be required for Gas1-induced growth arrest (5, 7).

p53 is a transcription factor (12, 29) able to transactivate genes containing its specific responsive element (10, 17, 20, 21) and able to repress TATA-mediated transcription (16, 25, 34). It is frequently mutated in many tumors, and most mutations are clustered in the central part of the protein, causing loss of the specific DNA binding properties and the consequent lack of induction of target genes (reviewed in reference 40). Overexpression of p53 in normal and in transformed cells induces a G₁ arrest (26) and, in certain cell types, programmed cell death (39). Evidence that the enhanced levels of p53 observed when cells are exposed to a wide variety of DNA-damaging agents are required for the G₁ arrest necessary for DNA repair (19, 24) suggests that p53 plays a crucial checkpoint function. Among the p53-induced genes, *waf1* is probably one of the critical downstream elements responsible for G₁ arrest (10). Waf1/p21, also identified as a potent inhibitor of cyclin-dependent kinases, Cip-1 (38), is a component of cdk/cyclin multi-protein complexes in normal cells. Activation of p53 with the subsequent accumulation of Waf1/p21 might be expected to block the activity of the cyclin-dependent kinases, resulting in

cell cycle block. Transcriptional activity, however, is not the only relevant function of p53. It has recently been demonstrated that transcriptional activation by p53 correlates with suppression of growth but is not sufficient for tumor suppression (3). Furthermore, two examples of p53-dependent apoptosis in the absence of transcriptional activation of target genes have been described (1, 35).

Here, we show that unscheduled expression of the plasma membrane *gas1* product induces growth suppression via wild-type p53. We also demonstrate that p53 does not rely on its intrinsic transactivation function to mediate the growth arrest induced by Gas1.

MATERIALS AND METHODS

Cell lines and culture conditions. The SV40 large T antigen (LTag) and all of the SV40 mutants were stably expressed in BALB/c 3T3 cells. BALB/c K1 cells express SV40 Tag-K1, which affects the binding to Rb. BALB/c 2809 and BALB/c 2811 express, respectively, SV40 Tag-2809 and SV40 Tag-2811, mutants that fail to bind p53 (41, 43).

The BALB/c (10)1 cell line does not contain endogenous p53 (15); the BALB/c Val5 cell line is a BALB/c (10)1 cell line stably expressing a p53 mutant, Val135, carrying a temperature-sensitive mutation at Val-135, which behaves as a mutant at 37°C and as the wild type at 32°C (14, 36). The BALB/c KH215 cell line is a BALB/c (10)1 cell line expressing the KH215 p53 mutant. To establish the BALB/c KH215 cell line, 6 × 10⁵ BALB/c (10)1 cells per 10-cm-diameter petri dish were transfected with 10 µg of pMSVKH215 (13) plasmid together with 1 µg of pSV2-Neo by the calcium phosphate procedure (31). Stable transfectants were selected by culture in medium containing 0.5 mg of G418 per ml for 2 weeks. Clones were picked, expanded, and checked for expression of p53. BALB/c Val135 25-26 is a BALB/c (10)1 cell line stably expressing the murine p53 mutant containing the temperature-sensitive Val-135 mutation (mutant Val135) and two amino-terminal mutations, L to Q at position 25 and W to S at position 26, corresponding to positions 22 and 23 of the human p53 (22). All of the cell lines used were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin-streptomycin (100 U and 100 µg/ml, respectively) and 2 mM glutamine.

Plasmids. pGDSV7-*gas1* and pGDSV7-hu-TR have been previously described (6, 7). pC53SN3 contains the human wild-type p53 under control of the cytomegalovirus (CMV) promoter (8). To construct pCMV-*gas1*, pC53SN3 was digested with *Bam*HI and the wild-type p53 was replaced with the murine *gas1* cDNA inserted as a *Bam*HI fragment.

RT-PCR amplification and sequencing. To perform a sequence analysis of p53

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expressed in BALB/c cell lines expressing LTag mutants, 1 μ g of total RNA extracted from cell lines was transcribed by Bethesda Research Laboratories reverse transcriptase (RT) with the buffers supplied in a final volume of 20 μ l. Amplification of the region between amino acids (aa) 99 and 283 was performed as described previously (15). PCR mixture was assembled in a final volume of 100 μ l by addition of the following reagents: 10 μ l of 10 \times PCR buffer (500 mM KCl, 200 mM Tris [pH 7.4], 25 mM MgCl₂, 1 mg of bovine serum albumin per ml), 20 pmol of 5' and 3' primers (5'-GCGAATTCCAACATATGGCTTCCACCT-3' and 5'-TACAGAAGAAGAAAATTGAATTCGC-3', respectively), 2.5 μ l of 10 mM deoxynucleoside triphosphates (dNTPs), and 5 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). The cDNA was amplified by 33 cycles (1 min at 94°C, 2 min at 55°C, and 3 min at 72°C). Amplified products were subcloned in Bluescript (Stratagene), and at least eight independent clones from each amplification were sequenced on both strands by the dideoxy-termination method (32).

Transfections and CAT assays. NIH 3T3 cells were transfected by the calcium phosphate procedure. A total of 2×10^5 cells were plated on 6-cm-diameter petri dishes, and 6 h later, the DNA precipitate (0.4 ml) was added. A total of 2.5 μ g of pG13-CAT plasmid, containing the p53-responsive element cloned upstream of the chloramphenicol acetyltransferase (CAT) reporter gene (21), was cotransfected with 2.5 μ g of pC53SN3 (8) or with 5 or 10 μ g of pCMV-gas1. The empty vector pCMV-Neo (8) was used as the carrier DNA to adjust the final concentration of DNA to 30 μ g/ml. After 12 h, the precipitate was replaced with DMEM containing 10% FCS. To check the efficiency of transfection and to monitor the percentage of cells in S phase, 6 h after the precipitate was removed, cells were trypsinized. One-fifth of the cells were plated on coverslips, and the remaining cells were reseeded on 6-cm-diameter petri dishes. Eighteen hours later, cells grown on coverslips were pulsed for 2 h with 50 μ M bromodeoxyuridine (BrdU), fixed, and processed for immunofluorescence. For the CAT assay, cells, after the washing of the DNA precipitate, were left in culture for 48 h and were assayed for CAT activity by routine procedures.

Western blotting (immunoblotting). Cellular lysates were prepared by addition of 2 \times sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 100 mM β -mercaptoethanol, 60 mM Tris [pH 6.8]) to the petri dish containing the relevant cells, and this mixture was then transferred to an Eppendorf tube. This was followed by vigorous mixing and boiling for 5 min. Protein lysates resolved by electrophoresis on an SDS-12% polyacrylamide gel were then transferred to a 0.2- μ m-pore-diameter nitrocellulose membrane by a semidry blotting apparatus (Bio-Rad) (transfer buffer: 20% methanol, 48 mM Tris, 39 mM glycine, and 0.0375% SDS). After being stained with Ponceau S, the nitrocellulose sheets were saturated for 2 h in BLOTTO-Tween 20 (50 mM Tris-HCl [pH 7.5], 500 mM NaCl, 5% nonfat dry milk, and 0.1% Tween 20) and incubated overnight at room temperature with Pab421 antibody (1:10 in BLOTTO-Tween). After three washes in BLOTTO-Tween 20, nitrocellulose was incubated for 1 h at room temperature with alkaline phosphatase-conjugated goat anti-mouse antibody (Dako). The Western blot was then developed with a chemiluminescence detection kit (Tropix) as recommended by the vendor.

Immunoprecipitation. For conformational analysis of p53, cells grown in DMEM containing 10% FCS were incubated for 30 min in methionine-free medium and then labelled with 200 μ Ci of [³⁵S]methionine per ml (1,000 Ci/ml [Amersham]) for 2 h. Cells were washed twice with phosphate-buffered saline (PBS) and lysed with 150 mM NaCl-50 mM Tris-HCl [pH 7.5]-5 mM EDTA-1% Nonidet P-40 in the presence of 1 mM phenylmethylsulfonyl fluoride and 10 μ g of chymostatin, leupeptin, antipain, and pepstatin per ml. After 30 min on ice, lysates were precleared by incubation with preimmune rabbit serum and Staph A Immunoprecipitin (Gibco-BRL) with gentle rocking. After 30 min at 4°C, lysates were centrifuged for 5 min in an Eppendorf centrifuge. An additional incubation with Staph A Immunoprecipitin (30 min at 4°C) was performed, and the lysates were centrifuged in an Eppendorf centrifuge for 5 min. Equal amounts of trichloroacetic acid-precipitable counts were immunoprecipitated with Pab421, Pab246, or Pab240 monoclonal antibodies for 1 h on ice. The immunocomplexes were collected with protein G-Sepharose (Pharmacia) and resolved by electrophoresis on an SDS-12% polyacrylamide gel.

Anti-murine Waf1/p21 antibody. Anti-Waf1/p21 antibody was raised against the peptide CLVSEPEDSPGGPGTSQGRKR, representing aa 117 to 137 of mouse Waf1/p21. The peptide was conjugated to keyhole limpet hemocyanin (Sigma) by glutaraldehyde (Sigma). The immunogen was injected into a rabbit. The resulting sera were affinity purified by being passed through a column made with the same peptide conjugated to Pierce Sulpholink as recommended by the vendor.

Microinjection and immunofluorescence. Cells cultured in DMEM containing 10% FCS were grown on coverslips (10⁴ cells per cm²) in 35-mm-diameter petri dishes. After 24 h of incubation at 37°C in a 5% CO₂ atmosphere, cells were microinjected with pGDSV7-gas1 (100 ng/ml) or pGDSV7-hu-TR (100 ng/ml) by the Automated Injection System (Zeiss, Oberkochen, Germany) as described previously (7) and left for 18 h in DMEM supplemented with 10% FCS. DNA synthesis assays were performed by addition of 50 μ M BrdU to the culture medium for 6 h before fixation. Cells were fixed and processed as described in reference 7. Unless otherwise specified, cells were labelled from the 18th to the 24th h after microinjection and then fixed in paraformaldehyde (3% in PBS) at room temperature. After 20 min, the coverslip was washed with PBS, and the cells were incubated for 5 min in 0.1 M glycine-PBS. Permeabilization was

performed with 0.1% Triton X-100 in PBS for 4 min. To reveal the incorporated BrdU, the coverslip was treated with 50 mM NaOH for 10 s and immediately washed three times with PBS. Incubation with antibodies was performed at 37°C in a wet chamber for 45 min. Gas1 was revealed by an affinity-purified anti-Gas1 rabbit antibody (7), followed by a goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugated antibody (Sigma). BrdU was detected by a specific anti-BrdU (immunoglobulin G2a [IgG2a]) (Amersham) monoclonal antibody, followed by a goat anti-mouse IgG2a rhodamine isothiocyanate (RITC)-conjugated antibody (Southern Biotechnology). hu-TR was recognized by the monoclonal antibody OKT9 (IgG1), followed by goat anti-mouse IgG1 FITC-conjugated antibody (Southern Biotechnology). p53 was detected with the mouse monoclonal antibody Pab240, followed by goat anti-mouse IgG1 FITC-conjugated antibody (Southern Biotechnology). Waf1 was detected by the affinity-purified anti-peptide antibody, followed by goat anti-rabbit RITC (Southern Biotechnology). Cell nuclei were stained with Hoechst 33342 (2 μ g/ml) in PBS for 2 min, followed by three washes in PBS. The coverslip was then mounted with Mowiol mounting medium on a microscope slide and observed in a Zeiss microscope with the appropriate fluorescence filters.

Color images were overlaid with a Zeiss confocal microscope.

RESULTS

Gas1 requires endogenous p53 but not Rb to suppress growth. Previous work suggested that either Rb (or Rb related) and/or p53 could be involved in Gas1-mediated growth arrest (7).

To better define the elements involved in the Gas1-induced growth suppression pathway, we used a panel of BALB/c 3T3 cell lines transformed with wild-type SV40 and various SV40 mutants containing mutations in the LTag that affect its ability to form complexes with Rb (Tag-K1) (18) and p53 (Tag-2809 and Tag-2811) (43). Figure 1A shows a schematic representation of the mutants used. The Tag-K1 mutant contains a point mutation in the large T protein at aa 107 converting a Glu to Lys (18) and fails to bind Rb (4). The two mutants Tag-2809 and Tag-2811 have linker insertion mutations at aa 409 (mutant 2809) and aa 424 (mutant 2811) (42). Both mutants encode Tags unable to interact with p53 (43). These cell lines were used as the recipients for ectopic expression of Gas1 by microinjection of *gas1* cDNA cloned in an SV40-driven vector lacking a functional SV40 origin (6, 7).

Asynchronously growing cells were microinjected with Gas1 or with the human transferrin receptor (hu-TR) used as a negative control. Growth suppression in microinjected (anti-Gas1-positive) cells was measured by scoring the relative fraction of cells in S phase (anti-BrdU positive) with respect to adjacent uninjected cells. As can be seen from the representative field in Fig. 1B and from the results reported in Table 1, only cells transformed with Tag mutants unable to bind p53 (Tag-2809 and Tag-2811) were significantly suppressed in entering the S phase by Gas1 overexpression. In contrast, cells transformed with the Tag-K1 mutant as well as with wild-type SV40, both preserving p53 binding, were not sensitive to the Gas1-induced inhibitory effect. Microinjection of the hu-TR cDNA cloned in the same vector did not show any significant effect. Similar results were also obtained using REF 52 rat fibroblasts transformed with the same Tag mutants as for the BALB/c cells (Table 1). To verify whether p53 was in the wild-type form in the cell lines used, immunoprecipitation analysis was performed with conformation-specific monoclonal antibodies. Figure 1C shows such an analysis and demonstrates that p53 is wild type, since it was recognized by Pab421 and Pab246 but not by Pab240. Furthermore, immunoprecipitations performed with Pab101, which is specific for Tag, confirmed coprecipitation of p53 with Tag only in the wild-type Tag and Tag-K1 BALB/c-transformed cell lines (not shown). Finally, sequence analysis of the RT-PCR-amplified region between aa 99 and 283 of p53 confirmed that p53 was wild type (data not shown).

Effect of Gas1 in cells not containing endogenous p53 or in cells expressing mutated p53. To analyze the role of p53 in a

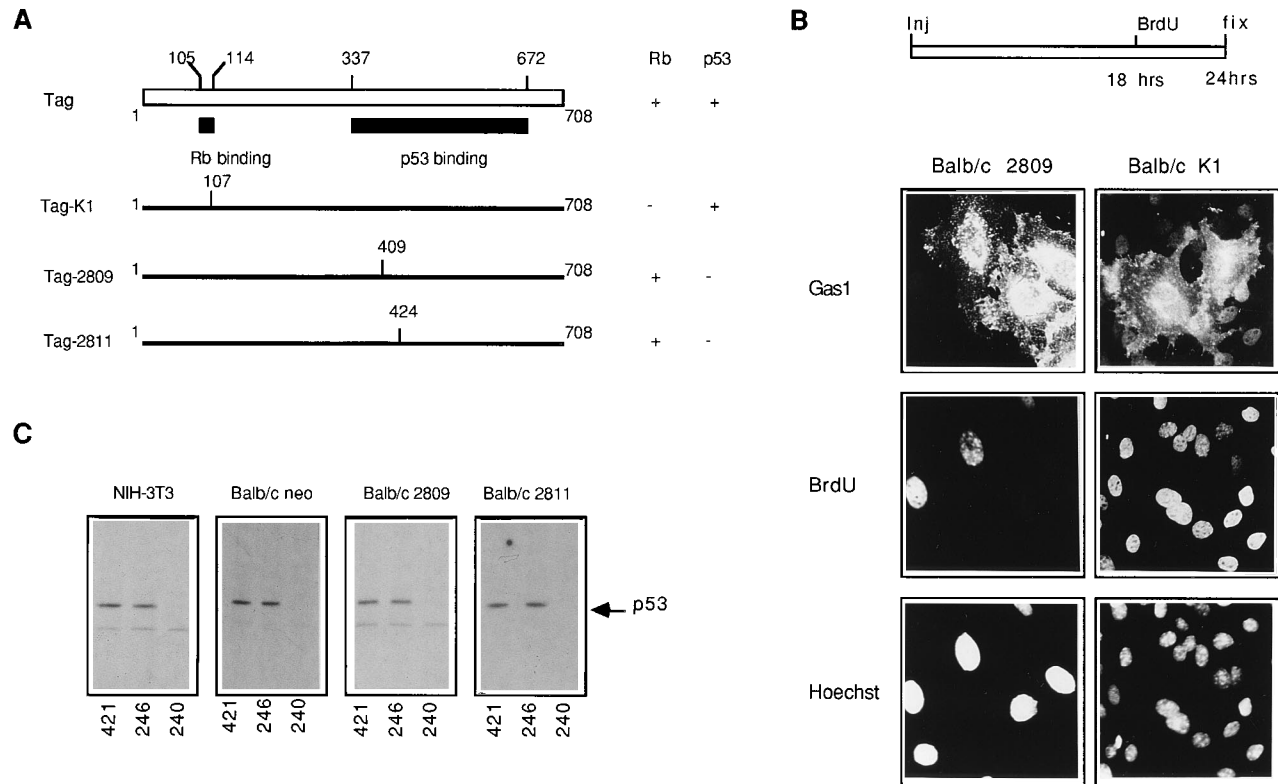


FIG. 1. Characterization of BALB/c 3T3 cell lines stably expressing the SV40 LTag mutants used for microinjection-driven Gas1 overexpression. (A) Schematic representation of SV40 LTag mutants used. Only the domains required to bind Rb and p53 are represented. (B) Schematic representation of the microinjection protocol and representative fields of BALB/c cells transformed with SV40 Tag mutants. Inj, injection. BALB/c 2809 cells are prevented from entering S phase by Gas1 overexpression, while BALB/c K1 cells are not inhibited. Microinjection and immunofluorescence were performed as described in Materials and Methods. Top panels anti-Gas1 staining; middle panels, anti-BrdU staining; bottom panels, Hoechst 33342 staining of nuclei. (C) Conformational analysis of p53 expressed in BALB/c 2809 and BALB/c 2811. Pab421 recognizes both wild-type and mutant p53. Pab246 recognizes only wild-type p53, while Pab240 recognizes only mutant p53.

more defined biological system, Gas1 was overexpressed in cells that do not contain endogenous p53 or in cells expressing mutant p53. We used the BALB/c (10)1 cells, in which both p53 alleles have been lost during the immortalization process (15), and the same cells engineered to constitutively express two p53 mutants: the temperature-sensitive p53 Val135 (BALB/c Val5), which behaves as a mutant at 37°C (36), or the KH215 mutant (13). In Fig. 2A, Western blot analysis shows the relative steady-state level of p53 expression in these three cell lines. As expected, BALB/c (10)1 cells do not express any p53 form recognizable by the Pab421 monoclonal antibody, while

both the Val135 and KH215 mutations are detected by the same antibody. When Gas1 was overexpressed by microinjection, no inhibition of S phase entry was observed in any of these cell lines (Fig. 2B), as similarly observed for the control hu-TR. In contrast, the parental (10)1 cell line was clearly responsive to microinjection-mediated overexpression of wild-type p53, resulting in a significant block in BrdU incorporation (Fig. 2B).

Similar analysis was also performed with fibroblasts derived from the p53-null mice (23). To assess the effect of Gas1 during cell cycle reentry, p53^{-/-} fibroblasts were serum starved for

TABLE 1. Growth suppression by Gas1 in cell lines expressing various Tag mutants^a

Cell line	No. of injected cells analyzed	% (mean ± SD) of BrdU in:		% (mean ± SD) relative inhibition ^b
		Injected cells	Uninjected cells	
BALB/c SV40 wild type	203	60.0 ± 8.1	60.2 ± 7.4	0.0 ± 4.1
BALB/c 2809	244	36.0 ± 4.0	65.2 ± 2.1	45.0 ± 6.2 (6.3 ± 5.4)
BALB/c 2811	206	17.0 ± 0.0	35.5 ± 3.5	51.5 ± 4.9 (2.3 ± 2.9)
BALB/c K1	232	52.0 ± 9.9	52.3 ± 5.7	1.0 ± 11.7
BALB/c Neo	146	30.3 ± 3.8	71.3 ± 2.6	59.3 ± 6.9 (9.7 ± 2.5)
REF 52 2811	296	21.0 ± 1.0	58.0 ± 7.6	63.3 ± 6.4 (4.7 ± 5.3)
REF 52 K1	443	49.3 ± 2.5	53.3 ± 4.6	7.2 ± 5.2

^a For details about the assay methods, see Materials and Methods.

^b The percentage of relative inhibition of DNA synthesis in injected cells was calculated by the following formula: % relative inhibition = [% of BrdU-positive cells (uninjected) - % of BrdU-positive cells (Gas1 positive)]/% of BrdU-positive cells (uninjected). Values in parentheses represent relative inhibition in cells microinjected with control DNA hu-TR.

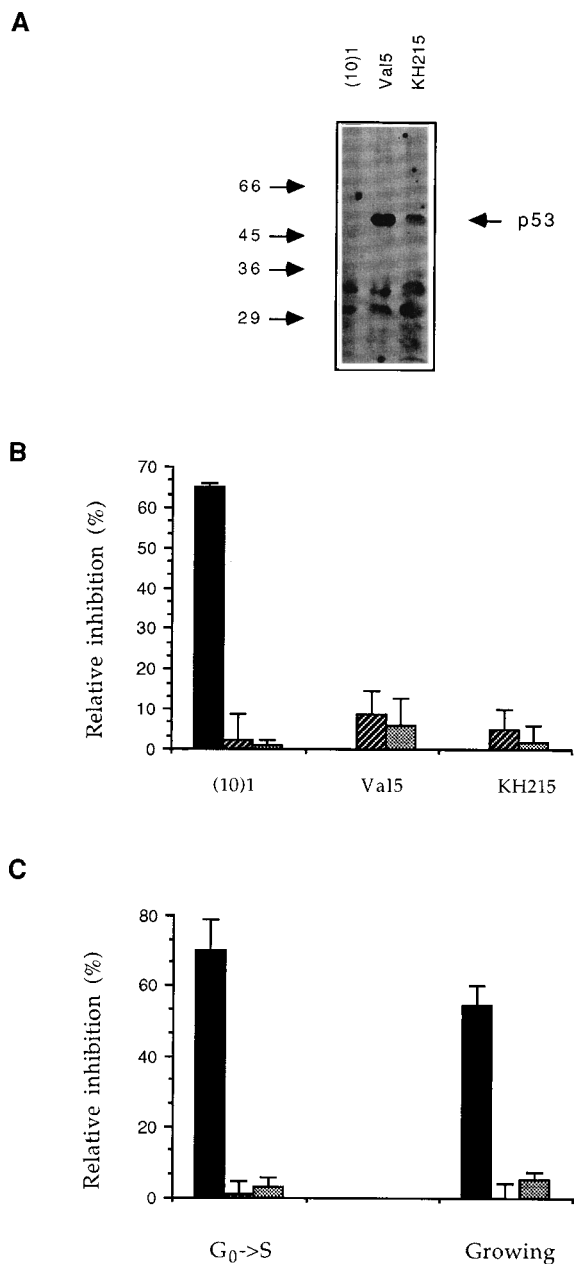


FIG. 2. (A) Western blot analysis of p53 expression in cell lines containing mutant p53 as revealed by monoclonal antibody Pab421. BALB/c (10)1 does not contain endogenous p53; Val5 cell line is a BALB/c (10)1 mutant stably expressing the p53 temperature-sensitive Val135 mutant; KH215 is a BALB/c (10)1 cell line stably expressing the p53 KH215 mutant. Arrows on the left are molecular mass markers (kilodaltons). (B) Results from various microinjection experiments with the cell lines described above as recipients. pGDSV7-gas1 (100 ng/ μ l), pGDSV7-hu-TR (100 ng/ μ l), and pC53SN3 (25 ng/ μ l) were used for microinjection. Relative inhibition was calculated as described in the footnote to Table 1. The results represent the mean of at least three independent experiments. Error bars indicate standard deviations of the mean. Solid bars, p53; hatched bars, pGDSV7-gas1; shaded bars, pGDSV7-hu-TR. (C) Effect of Gas1 overexpression in fibroblasts derived from the p53-null mice either growing or during the G₀-to-S phase transition. Growing cells were microinjected according to the protocol described in the legend to Fig. 1. For the analysis of the effect of Gas1 during the G₀-to-S phase transition, p53^{-/-} fibroblasts were serum starved for 48 h in 0.1% FCS. Microinjection with 25 ng of pC53SN3, 100 ng of pGDSV7-gas1, or 100 ng of pGDSV7-hu-TR expression plasmids per μ l was performed at 48 h from starvation, and cells were left for a further 24 h in 0.1% FCS. After 72 h in 0.1% FCS, less than 5% of the nuclei incorporated BrdU after 2 h of labelling. Cells were finally stimulated with 10% FCS, together with 50 mM BrdU, for 18 h before analysis. The results represent the mean of at least three independent experiments. Error bars indicate standard deviations of the mean.

72 h in 0.1% FCS and microinjected with pGDSV7-gas1 or control vectors. Twenty-four hours after microinjection, cells were stimulated to enter S phase by addition of 10% FCS in the presence of BrdU. Overexpression of Gas1 was also induced in the same p53^{-/-} cells during the asynchronous cell cycle. No effect of Gas1 overexpression was observed when the relative fraction of cells in S phase under both conditions was measured (Fig. 2C). As expected, overexpression of wild-type p53 results in a significant block in BrdU incorporation under both conditions (Fig. 2C).

Taken together, these results demonstrate the requirement of wild-type p53 to mediate the effect of Gas1 in asynchronously growing cells as well as during the G₀-to-S phase transition.

Gas1 does not induce p53 transactivation function. p53 is a transcription factor (21), and increased levels of the protein cause G₁ arrest (27), as mediated by the induction of target genes such as *waf1* (9, 10).

Thus, we asked whether Gas1 growth arrest could involve the transcriptional activation function of p53. NIH 3T3 cells were thus transfected with pCMV-gas1 together with the pG13-CAT plasmid, which contains a p53 consensus-responsive DNA element linked to the CAT reporter gene (21). After transfection, cells were split for evaluation of CAT activity and expression of Gas1 combined with the analysis of in situ BrdU incorporation. As shown in Fig. 3A, no significant increase in CAT activity was observed in the cells cotransfected with pCMV-gas1 and pG13-CAT compared with either wild-type p53 (positive control) or vector alone (negative control). Results were comparable when increasing ratios of pCMV-gas1 with respect to pG13-CAT were used. Furthermore, when BrdU incorporation in the transfected Gas1-positive cells was analyzed, significant inhibition of S phase entry with respect to hu-TR control-transfected cells was evident, and it was comparable to the effect detected in wild-type p53-transfected cells (Fig. 3B).

We conclude that Gas1 growth-suppressive activity requires wild-type p53 but does not involve its sequence-specific transactivation functions.

Transcriptional activation function of p53 is not required for Gas1-induced growth arrest. To substantiate the previous results, we used a cell line expressing the p53 temperature-sensitive Val135 mutant engineered to contain two additional mutations at aa 25 and 26. These two codons represent the respective mutations at aa 22 (Leu to Gln) and aa 23 (Trp to Ser) introduced in human p53 as previously described to specifically inhibit the transactivation function of p53 while leaving other functions, such as DNA binding and protein oligomerization, unaltered (22). This mutant p53 product, because of the temperature-sensitive mutation, regains its ability to bind DNA and to oligomerize when exposed to the permissive temperature (32°C), but it still lacks the transactivation function (22a).

A BALB/c(10)1 cell line stably expressing the temperature-sensitive Val135 p53 25–26 mutant (BALB/c Val135 25–26) was analyzed for its properties of transcriptional activity as related to the p53-dependent G₁-to-S phase block. The BALB/c Val5 cell line, expressing the temperature-sensitive Val135 p53 mutant, was used as a positive control. Both lines were analyzed at the nonpermissive (37°C) and permissive (32°C) temperatures for BrdU incorporation and expression of *Waf1/p21*. Figure 4 shows that BALB/c Val135 25–26 cells did not G₁-S arrest when transferred at 32°C. In fact, 24 h after the temperature shift down, 50% of these cells were still BrdU positive, whereas only 5% of the BALB/c Val5 cells incorporated BrdU (Fig. 4A, central panel, and B). Consistent with

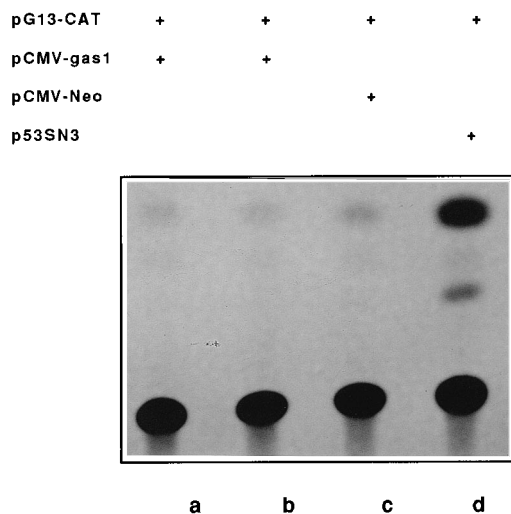
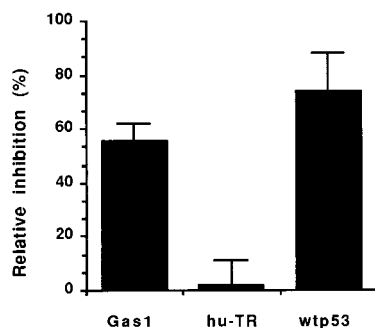
A**B**

FIG. 3. (A) Overexpression of Gas1 does not enhance the transactivating functions of wild-type p53. NIH 3T3 cells were transfected by the calcium phosphate procedure. A total of 2.5 μ g of pG13-CAT plasmid was cotransfected with 2.5 μ g of pC53SN3 (8) (lane d) or with 5 μ g (lane a) or 10 μ g (lane b) of pCMV-gas1. The empty vector pCMV-Neo (8) was used as a carrier DNA to adjust the final concentration of DNA to 30 μ g/ml. Lane c, cells transfected with 10 μ g of pCMV-Neo. (B) Inhibition of S phase in the positive transfected cells relative to the surrounding nontransfected cells. Six hours after removal of the calcium-phosphate DNA precipitate, cells were trypsinized. One-fifth of the cells were plated on coverslips, and the remaining cells were reseeded on 6-cm-diameter petri dishes for CAT assays. Eighteen hours later, cells grown on coverslips were pulsed for 2 h with 50 μ M BrdU and then were fixed and processed for immunofluorescence. Results represent the mean of three independent experiments in which at least 400 Gas1-overexpressing cells were scored. wt, wild type.

this result, the BALB/c Val135 25–26 mutant also failed to show increased levels of Waf1/p21, as revealed by either immunofluorescence or Western blot analysis (Fig. 4A, lower panel, and C). Waf1 was instead clearly visible in the nuclei of BALB/c Val5 cells grown at 32°C (Fig. 4A, lower panel) as well as by Western blot analysis (Fig. 4C).

To evaluate whether Gas1 was able to block cell proliferation in the BALB/c Val135 25–26 cell line at the permissive temperature, cells were microinjected with the pGDSV7-gas1 expression vector and kept at either 37°C or 32°C. As can be seen from the representative field of microinjected cells in Fig. 5 and from the results from the histogram shown in Fig. 5c, the BALB/c Val135 25–26 cells overexpressing Gas1 were significantly inhibited in S phase entry only at 32°C but not at 37°C.

These results strongly support evidence that the transactivation function of p53 is not required to mediate Gas1 growth suppression.

Waf1 is not required for p53-mediated Gas1 growth arrest. Since the temperature-sensitive p53 25–26 mutant is able to bind DNA at 32°C (22), it was possible that, in the presence of Gas1, this p53 mutant could interact with other transcription factors and still be able to induce specific targets such as *waf1*. Since BALB/c Val135 25–26 cells are incapable of upregulating Waf1/p21 when transferred at 32°C, we therefore asked whether increased levels of Waf1/p21 could be specifically detected when Gas1 was overexpressed in the same cells at 32°C. Microinjection-driven overexpression of Gas1 was performed either at 37°C or at 32°C in BALB/c Val135 25–26 and in BALB/c Val5 used as a positive control. Since both anti-Gas1 and anti-Waf1 antibodies are rabbit polyclonal antibodies, pGDSV7-gas1 was mixed in a 4:1 ratio with pGDSV7-hu-TR DNA as an expression marker in order to recognize microinjected cells through a hu-TR-specific monoclonal antibody. Figure 6 shows that Waf1/p21 was clearly increased in the BALB/c Val5 cell line either in the microinjected or in the surrounding cells at the permissive temperature (Fig. 6b). Conversely, in the BALB/c Val135 25–26 mutant, Waf1/p21 accumulation at 32°C was not observed most specifically in the microinjected cells (Fig. 6d).

We could thus conclude that Gas1 is able to induce growth arrest via a transactivation-deficient p53 and that Waf1/p21 is not involved in this growth arrest mechanism.

DISCUSSION

We have previously demonstrated that overexpression of Gas1 in quiescent fibroblasts prevents reentry into the cell cycle (7). Gas1's growth-suppressive effect is also maintained in asynchronously growing NIH 3T3 cells and in many oncogenically transformed murine cell lines (7) as well as in several human-derived tumor cells (5). Some cell lines were, however, refractive to ectopic expression of Gas1 (5, 7). A common feature of these cells was the absence of functionally active Rb and p53 protein products because of either formation of a protein complex with a DNA viral oncogene product or genetic lesions. This evidence thus indicated that p53 and/or Rb could serve as a potential downstream effector for the Gas1-induced growth arrest.

To more specifically establish which of the oncosuppressor proteins was involved in Gas1-induced growth arrest, we used a panel of murine cell lines transformed with SV40 LTag mutants (41, 43) that are unable to bind either Rb (18) or p53 (41, 43) protein products. These mutants were previously employed to analyze the roles of Rb and p53 in immortalization and transformation of murine fibroblasts by SV40 LTag (41, 43).

When Gas1 was ectopically expressed in this panel of cell lines, significant growth suppression was detected only in those lines harboring LTag mutants that fail to form complexes with p53. Thus, in these cells, the presence of endogenous wild-type p53 freed from interaction with SV40 Tag was seemingly sufficient to reestablish the growth arrest induced by Gas1 overexpression. This result was consistently demonstrated in a more defined cellular system with cell lines that lack both p53 alleles. The immortalized BALB/c (10)1 cell line, which has spontaneously lost both p53 alleles (15) as well as fibroblasts derived from p53 $-/-$ mice (25), failed to arrest growth after ectopic expression of Gas1 both in asynchronously growing cultures and during synchronous G₀-to-S phase transition. When p53 mutant alleles (Val135 and KH215) were constitu-

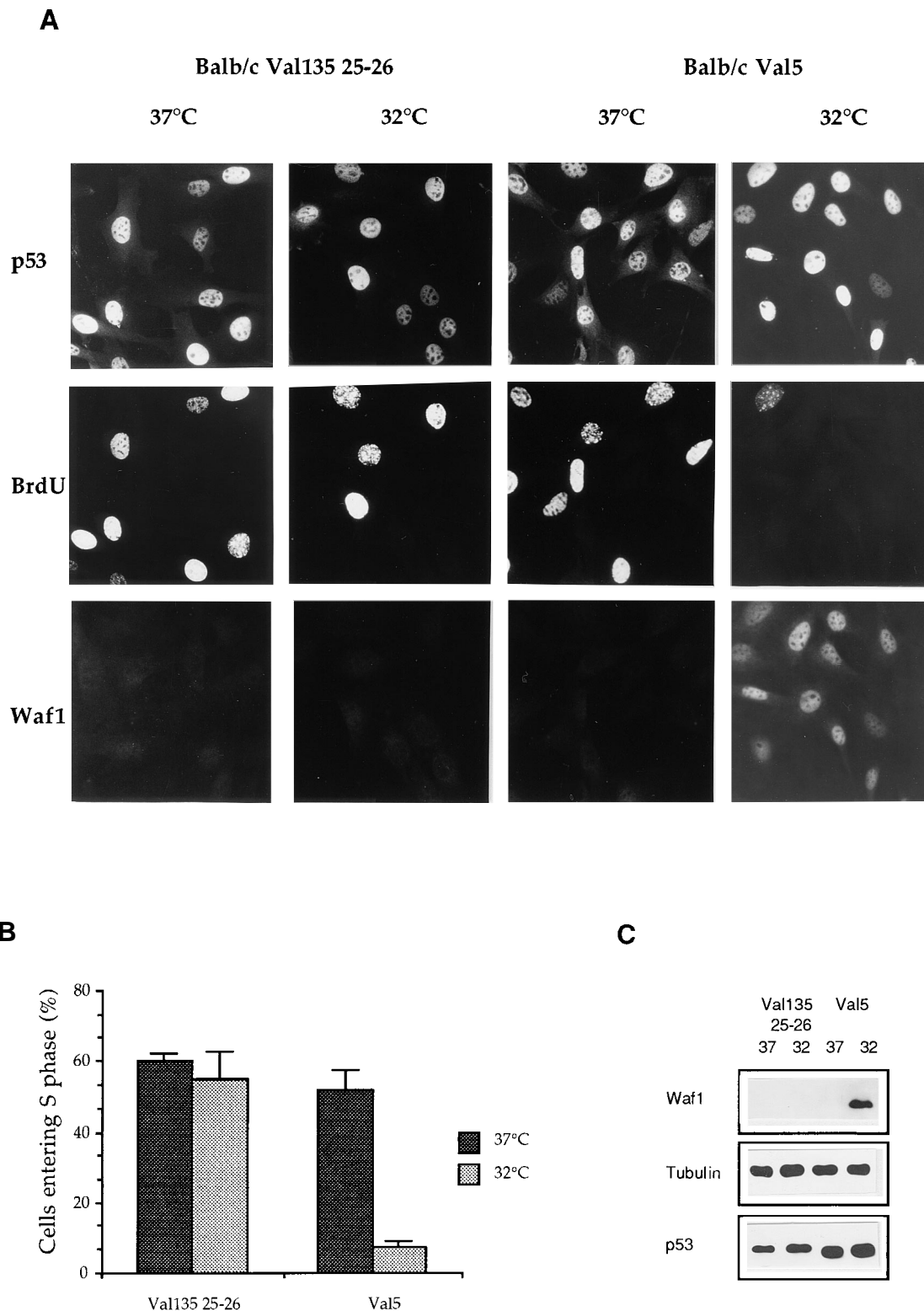


FIG. 4. Analysis of p53 function in BALB/c Val135 25-26 and BALB/c Val5 cell lines grown at the permissive (32°C) or nonpermissive (37°C) temperature. (A) BALB/c Val135 25-26 and BALB/c Val5 cells were either kept at 37°C or transferred at 32°C. After 24 h, 50 μ M BrdU was added to the culture medium for 1 h. Cells were then fixed and processed for triple immunofluorescence. (Top panels) p53 was detected with Pab240 monoclonal antibody (IgG1) followed by goat anti-mouse IgG1-FITC. (Middle panels) BrdU was recognized by the anti-BrdU monoclonal antibody (IgG2a) followed by goat anti-mouse IgG2a-RITC; (Bottom panels) Waf1 was detected by anti-peptide antibody followed by goat anti-rabbit biotin and streptavidin-aminomethylcoumarin (AMCA) (B) Percentage of BALB/c Val135 25-26 cells in S phase at 37°C or 32°C compared with that of BALB/c Val5 cells under the same experimental conditions. (C) Western blot analysis of Waf1 and p53 (Pab421) expression in the BALB/c Val135 25-26 and BALB/c Val5 cell lines at 37 or 32°C. Equal amounts of protein were loaded as assessed by performing a separate Western blot with anti-tubulin monoclonal antibody.

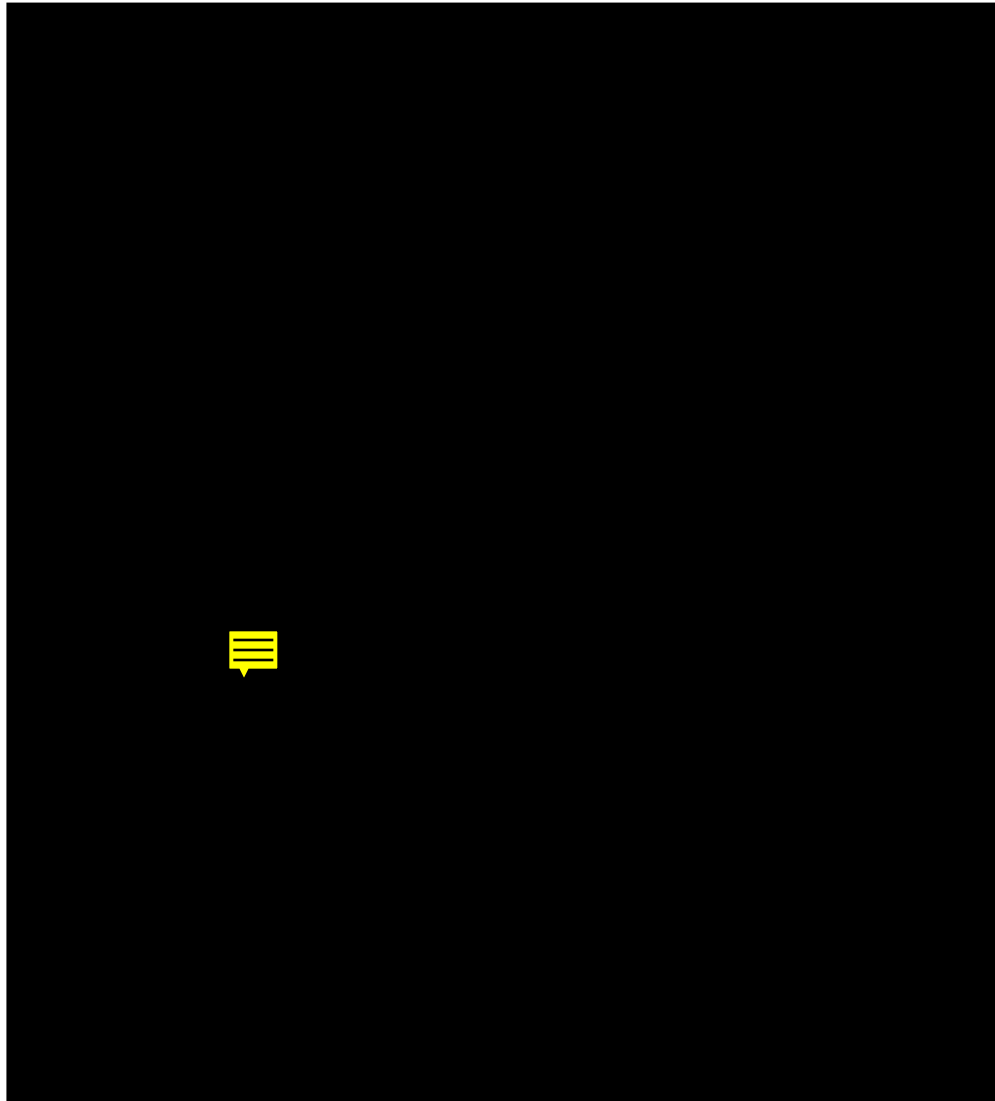


FIG. 5. (a and b) Representative field of BALB/c Val135 25–26 cells injected with pGDSV7-gas1 at 37°C (a) or 32°C (b). Green staining, Gas1; red staining, BrdU in the same cells. (c) Growth suppression by Gas1 in BALB/c Val135 25–26 cells expressing the p53 amino-terminal mutant. Cells were cultured in DMEM supplemented with 10% FCS. For microinjection, cells were seeded on coverslips, and 24 h later, they were either left at 37°C or transferred to 32°C for a further 24 h. They were then microinjected with 100 ng of pGDSV7-gas1 or pGDSV7-hu-TR as a negative control per μ l. Eighteen hours after microinjection, cells were labelled with BrdU for 6 h and then processed for immunofluorescence. The results show the mean of three independent experiments. Relative inhibition was calculated as described in the footnote to Table 1.

tively expressed in the BALB/c (10)1 cell line, Gas1 overexpression was again unable to block cell proliferation. We therefore conclude that wild-type p53 is required to mediate the Gas1 growth arrest effect.

p53 is a transcription factor which plays a critical role as a mediator of cellular response to environmental perturbations. An increased level of p53, such as after DNA damage, induces the transcription of several target genes possibly responsible for the p53 downstream effects: growth arrest or apoptosis. These effects, however, appear to be cell type specific, since fibroblasts mainly respond with G_1 arrest while lymphoid cells respond with apoptosis (2, 20, 23, 37, 40). The transactivation function of p53 is required for the p53-induced cell cycle block (3, 28), and at least one of the p53-induced genes, *waf1*, seems to be required for the G_1 arrest (9, 10). We thus wanted to test whether the involvement of p53 in cell cycle block induced by Gas1 also required the transcriptional functions of wild-type

p53. Cotransfection of pCMV-gas1 and the pG13-CAT plasmid, which contains the p53 recognition sequence, was therefore performed with NIH 3T3 cells expressing wild-type p53. Although the transfected Gas1-positive cells were efficiently growth arrested, we could not detect any increase in CAT activity, thus indicating that growth arrest induced by Gas1 was not able to regulate the transactivation functions of p53 in NIH 3T3 cells. This result could be explained, however, by assuming that overexpression of Gas1 failed to increase the amount of endogenous p53 to the levels required for transactivation of the exogenous CAT reporter but was still sufficient to induce transcription of endogenous p53-responsive genes such as *waf1*.

To more clearly define whether the transactivation functions of p53 were required or dispensable for the Gas1-induced growth arrest, a cell line (BALB/c Val135 25–26) constitutively expressing a p53 mutant defective in the transactivation func-

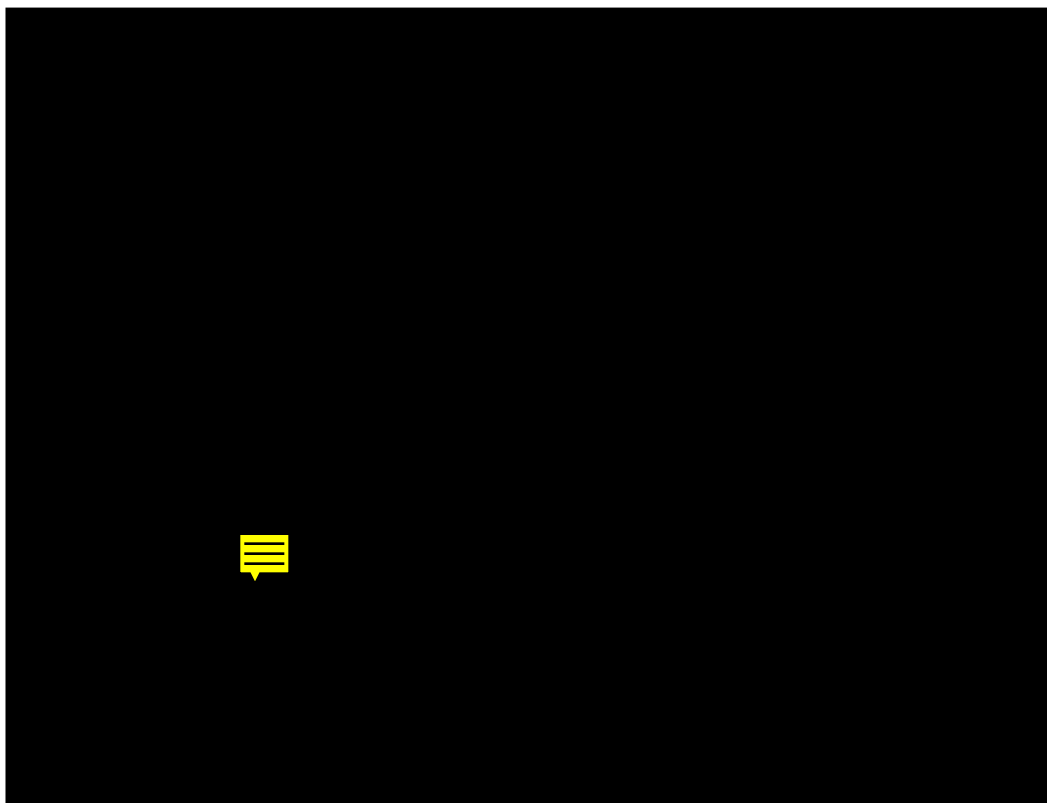


FIG. 6. Microinjection of Gas1 in BALB/c Val135 25–26 at 32°C is not accompanied by induction of Waf1/p21 compared with control BALB/c Val5. Cells were seeded on coverslips, and 24 h later, they were either left at 37°C or transferred to 32°C for a further 24 h. They were then microinjected, and after 24 h, they were fixed and processed for immunofluorescence. Because both anti-Gas1 and anti-Waf1 are rabbit polyclonal antibodies, 25 ng of pGDSV7-hu-TR per μ l was added to the 100 ng of pGDSV7-gas1 DNA sample per μ l as a marker (Gas1-TR/Waf1) for microinjection. Injected cells were thus revealed by the OKT9 monoclonal antibody to human transferrin receptor followed by goat anti-mouse IgG1-FITC; Waf1/p21 was revealed by rabbit anti-peptide antibody followed by goat anti-rabbit RITC. Green staining, hu-TR/gas1; red staining, Waf1/p21. (a) BALB/c Val5 at 37°C. (b) BALB/c Val5 at 32°C. (c) BALB/c Val135 25–26 at 37°C. (d) BALB/c Val135 25–26 at 32°C.

tions (22a) was used as a recipient for Gas1 ectopic expression. This p53 mutant contains a double mutation in the amino-terminal region (aa 25 and 26), constitutively abrogating its transactivation function, and a temperature-sensitive mutation at aa 135 (Val135) (26, 27), conferring temperature-dependent regulation to the central core-specific DNA binding region. Consistently, this BALB/c Val135 25–26 cell line fails to arrest growth or is incapable of increasing Waf1/p21 levels at the permissive temperature (32°C). When Gas1 was ectopically expressed in this cell line at 32°C, significant growth arrest was achieved, however. Again, it was possible that at 32°C, the temperature-sensitive p53 25–26 mutant having unaltered DNA binding properties could still interact with other transcription factors, thereby inducing the same p53 downstream targets, including Waf1/p21. This possibility was ruled out, however, since we could not detect induction of Waf1/p21 when Gas1 was overexpressed at 32°C in the BALB/c Val135 25–26 cells.

We can therefore conclude that the unscheduled expression of a typical growth arrest-associated gene in actively dividing fibroblastoid cell lines induces a wild-type p53-dependent growth arrest that is both mechanistically and phenotypically distinct from the G₁ arrest associated with the transactivation function of p53.

Recently it has been demonstrated that the transactivation function of p53 is not required for induction of apoptosis (1, 35), leading to the hypothesis that the relevant mechanisms by

which p53 induces apoptosis could be distinct from the mechanisms that mediate the transactivation-dependent G₁ arrest. In this context, the transcriptional repressor function of p53 has been proposed (1) to play a critical role in mediating p53-dependent apoptosis. Consistently, the adenovirus E1B 19K protein has been shown to modulate both p53-mediated transcriptional repression and apoptosis (30). Therefore, we speculate that *gas1* overexpression specifically modulates the p53 transcriptional repression function, leading to growth suppression as a default pathway in a cellular context that is less susceptible to p53-dependent apoptosis. In fact, we have never detected signs of apoptosis in all of the cellular systems analyzed for *gas1* overexpression either by in situ DNA fragmentation or by nuclear and/or cellular morphological phenotypes. Changes in cellular morphology were instead evidently noticeable after similar overexpression of the Gas3 membrane protein, recently established to induce a typical apoptotic phenotype (11). In addition, the relative number of Gas1-positive-overexpressing cells recovered with respect to the total number of cells microinjected with the relative plasmid DNA has been consistently similar to the control hu-TR and has never been significantly reduced, as in the case of Gas3 overexpression (11). Alternatively, it is possible that a Gas1-overexpression-dependent signalling pathway could modulate the interaction of wild-type p53 with other proteins, thus resulting in growth suppression.

Notwithstanding the elucidation of the molecular mecha-

nism responsible for the effect of Gas1 overexpression on p53, it obviously remains to be understood whether the Gas1-dependent growth arrest has any physiological importance in regulating G_0 with respect to other types of growth arrest.

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The first two authors equally contributed to this work.

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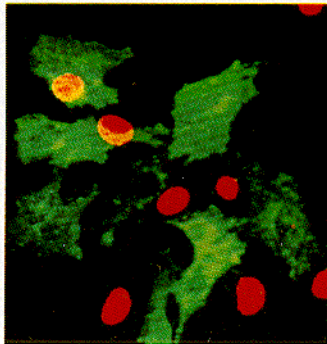
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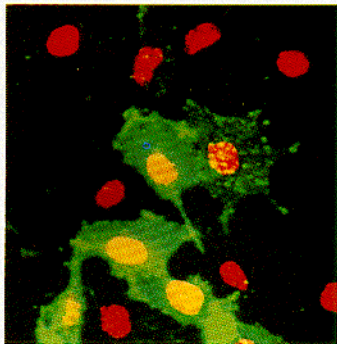
32°C

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a



b

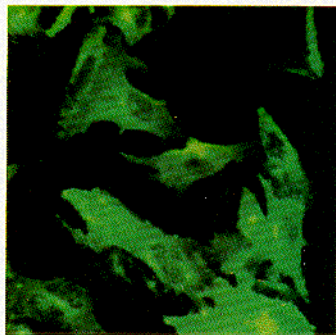


Gas1/BrdU

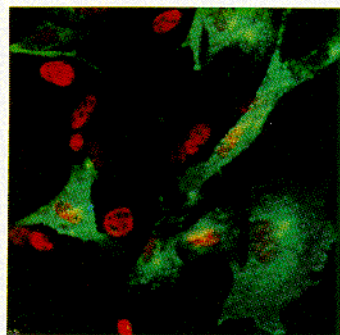
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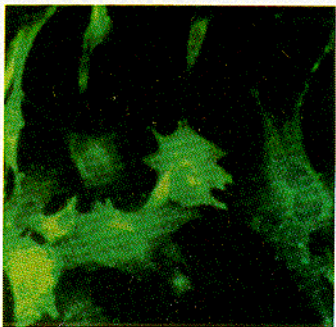
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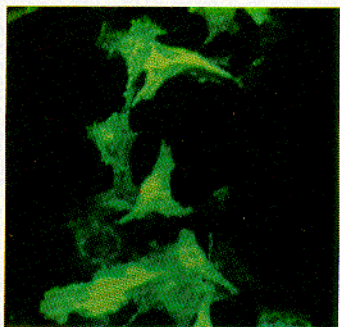
Val5

Gas1-TR/Waf1

c



d



Val135 25-26

Gas1-TR/Waf1