Title: Restoration of endogenous wild-type p53 activity in a glioblastoma cell line with intrinsic temperature-sensitive p53 induces growth arrest but not apoptosis.

Authors: Ikeda J, Tada M, Ishii N, Saya H, Tsuchiya K, Okaichi K, Mishima K, Sawamura Y, Fulci G, Liu TJ, Van Meir EG

Journal: International journal of cancer

Year: 2001 Oct 1

Volume: 94

Issue: 1

Pages: 35-43

DOI: 10.1002/ijc.1431

Published in final edited form as:
Int J Cancer. 2001 October 1; 94(1): 35. doi:10.1002/ijc.1431.

RESTORATION OF ENDOGENOUS WILD-TYPE p53 ACTIVITY IN A GLIOBLASTOMA CELL LINE WITH INTRINSIC TEMPERATURE-SENSITIVE p53 INDUCES GROWTH ARREST BUT NOT APOPTOSIS

Jun Ikeda[1], **Mitsuhiro Tada**[1,2,*], **Nobuaki Ishii**[1], **Hideyuki Saya**[3], **Kazuhiko Tsuchiya**[4], **Kumio Okaichi**[5], **Kazuhiko Mishima**[6], **Yutaka Sawamura**[1], **Giulia Fulci**[7,8], **Ta-Jen Liu**[9], and **Erwin G. Van Meir**[7,8]

[1]Department of Neurosurgery, Hokkaido University Graduate School of Medicine, Sapporo, Japan [2]Division of Cancer-Related Genes, Institute for Genetic Medicine, Hokkaido University, Sapporo, Japan [3]Department of Tumor Genetics and Biology, Kumamoto University School of Medicine, Kumamoto, Japan [4]Department of Radiology, Hokkaido University Graduate School of Medicine, Sapporo, Japan [5]Department of Radiation Biophysics, Radiation Effect Research Unit, Atomic Bomb Disease Institute, Nagasaki University School of Medicine, Nagasaki, Japan [6]Department of Neurosurgery, University of Tokyo, Tokyo, Japan [7]Laboratory of Molecular Neuro-Oncology, Winship Cancer Institute and Department of Neurological Surgery, Emory University, Atlanta, GA, USA [8]Laboratory of Tumor Biology and Genetics, University Hospital (CHUV), Lausanne, Switzerland [9]Department of Neuro-Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA

Abstract

p53 protein is a transcription factor involved in multiple tumor-suppressor activities including cell cycle control and apoptosis. *TP53* gene is frequently mutated in glioblastoma, suggesting the importance of inactivation of this gene product in gliomagenesis. Restoration of p53 function in glioblastoma cell lines deficient for p53 has shown that p53 induces growth arrest or apoptosis depending on the cell line and vector used to transduce wild-type *TP53* alleles. Considering that astrocytes grow and express p53, it is not clear whether these results reflect physiologic responses or the result of p53 overexpression in combination with cellular responses to viral vector infection. Here, we reassessed this issue using a glioblastoma cell line (LN382) that expresses an endogenous temperature-sensitive mutant p53. This cell line expresses *TP53* alleles (100% as determined by a p53 transcriptional assay in yeast) mutated at codon 197 GTG (Val) > CTG (Leu). We found that the p53 protein in these cells acted as an inactive mutant at 37°C and as a functional wild-type p53 below 34°C as demonstrated by several lines of evidence, including (*i*) restoration of transactivating ability in yeast, (*ii*) induction of p53-modulated genes such as *CDKN1*^{p21} and transforming growth factor-α, (*iii*) disappearance of accumulated p53 protein in the nucleus and (*iv*) decrease in steady state p53 protein levels. This temperature switch allowed p53 levels, which were close to physiological levels to dramatically reduce LN382 cell proliferation by inducing a G_1/S cell cycle block, but not to induce apoptosis. The lack of apoptosis was considered to be a result of the low level p53 expression, because increasing wild-type p53 levels by adenoviral-mediated gene transfer

*Correspondence to: Division of Cancer-Related Genes, Institute for Genetic Medicine, Hokkaido University, N-15 W-7, Kita-Ku, Sapporo 060-8638, Japan. Fax: 181-11-706-7870. m_tada@med.hokudai.ac.jp.

caused apoptosis in these cells. The LN382 cell line will be extremely useful for investigations into the roles of p53 in cellular responses to a variety of stimuli or damages.

Keywords

p53; temperature-sensitive mutant; cell cycle; growth arrest

TP53 is the most frequently mutated gene in cancer and about 40% of glioblastoma express mutated p53 protein. The effects of many anti-cancer agents are largely dependent on p53-mediated apoptosis at the G_1/S cell cycle checkpoint subsequent to DNA damage. Therefore, *TP53* status may help stratifying patients into subsets that may respond differently to treatments such as chemo- and radiotherapy.[1,2] Since an inverse relationship between *TP53* mutation and radiosensitivity has been observed in glioblastomas,[3] it has been hypothesized that inactivation of *TP53* may confer resistance to radio- and possibly chemotherapeutic agents.[4,5] Clearly it is important to better understand the role of physiologic responses of p53 in these processes for developing optimal therapeutic strategies for glioma patients.

For studying the role of p53 in these mechanisms, wild-type (WT) p53 function was restored in various glioblastoma cell lines by several gene transfer methods, including stable transfection of p53 constructs inducible by dexamethasone6 or tetracycline,7 an exogenous murine p53 Val 135 temperature-sensitive (ts) mutant,8 p53 expression from retroviruses9 and adenoviruses.10 Expression of p53 in glioblastoma cells from stably transfected clones or retrovirally transduced genes induced growth arrest or senescence, whereas expression from adenoviruses induced apoptosis. Reversibility of the growth arrest was not determined. The reasons for these uneven responses are unclear but may be linked to variable levels of p53 expression driven by the exogenous promoters used in all these constructs, perturbation of cellular status by the large difference of culture temperature (6°C) for the exogenous ts mutant, incomplete function of murine p53 in human cells, variation in p53 responses in different cell lines and synergy of p53 action with cellular responses to viral infection.

To reassess glioblastoma cell responses to restoration of WT p53 activity close to physiologic levels, we have taken advantage of our discovery of a glioblastoma cell line (LN382) expressing a p53 mutant with ts properties in yeast.[11] Here we demonstrate that this endogenous p53 mutant switches from mutant to WT activity over a narrow 3°C temperature range (37–34°C) in LN382 cells, using several assays for p53 activity. Using this cell line, we have subsequently analyzed changes in cell proliferation and apoptosis in response to p53 activation in the absence of genotoxic stress. The absolute advantage of using the intrinsic ts mutant over artificially transfected *TP53* genes is that because the ts mutant is under the control of endogenous *TP53* gene regulatory elements its expression level is expected to be in the physiologic range. Also, the results are not influenced by the selection of a particular clone resistant to cell-cycle arrest, senescence or apoptosis induced by p53, and does not undergo counterselection of mutant p53, which occurs rapidly after transfection of WT *TP53* alleles.[9,12] This cell line thus will be invaluable in permitting biochemical analyses of the molecular mechanisms underlying p53 action in a variety of situations.

MATERIAL AND METHODS

Glioblastoma cell lines and culture conditions

Glioblastoma cell line U251MG was maintained with Eagle's minimal essential medium (MEM; Gibco-BRL, Grand Island, NY) supplemented with 0.1 mM L-glutamine, 0.1% $NaHCO_3$, 100 U/ml penicillin-G and 10% fetal calf serum (Gibco-BRL). LN382 cells were

maintained with DMEM (Gibco-BRL) with the same supplementation. They were cultured at 37°C in a humidified 5% CO_2 atmosphere.

p53 transcriptional assay in yeast

p53 transcriptional assay in yeast[13] and sequencing of p53 cDNA plasmids recovered from transfected yeast were performed as described elsewhere.[14] To test temperature sensitivity of the mutant p53 present in LN382 cells, yeast colonies were replated on SDA5HTαα plates[13, 14] and cultured at 30°, 34° or 37°C for 2–3 days.

Multiplex reverse transcription-PCR (RT-PCR)

Concentrations of the total RNA extracted from the cells were adjusted at 1 μg/μl and RNA integrity was verified by electrophoresis in a 1% agarose gel. Each RNA sample (1 μg) was reverse transcribed at 37°C for 1 hr in a total of 20 μl of the following reaction mixture: 50 mM Tris-HCl, 75 mM KCl, 7.5 mM dithiothreitol, 2.0 mM $MgCl_2$, 0.5 mM each deoxynucleotide-triphosphate (dNTP), 10 μg/μl random hexamer (pdN6) and 10 U/μl Moloney-murine leukemia virus (M-MLV) reverse transcriptase (Gibco-BRL). cDNA was amplified by PCR with 0.05 U/μl AmpliTaq Gold DNA polymerase (Perkin-Elmer, Chiba, Japan) on a Thermal Cycler Model 2400 (Perkin-Elmer) in a total of 25 μl of the following reaction mixture: 1.0 μl RT product, 10 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM each dNTP and each primer at the concentration described below. The sequence of the PCR reaction was an initial denaturation for 10 min at 95°C, followed by 28–30 cycles of denaturation for 40 sec at 94°C, annealing for 40 sec at 60°C and extension for 60 sec at 72°C. A final extension was performed for 5 min at 72°C. The 5′ to 3′ sequence of used primer pairs and their predicted amplicon sizes were for glyceraldehyde 3-phosphate dehydrogenase (G3PDH): 5′-TGA AGG TCG GAG TCA ACG GAT TTG GT-3′, 5′-CAT GTG GGC CAT GAG GTC CAC CAC-3′, 983 bp; for Bax: 5′-CAG GAT GCG TCC ACC AAG AAG C-3′, 5′-GGG TGA GGA GGC TTG AGG AGT C-3′, 347 bp; for p21: 5′-GGG GAA GGG ACA CAC AAG AAG-3′, 5′-GGG GAG CCG AGA GAA AAC AGT-3′, 480 bp; for inducible nitric oxide synthase (iNOS): 5′-CGG TGC TGT ATT TCC TTA CGA GGC GAA GAA GG-3′, 5′-GGT GCT GCT TGT TAG GAG GTC AAG TAA AGG GC-3′, 259 bp; for c-jun: 5′-GCA TGA GGA ACC GCA TCG CTG CCT CCA AGT-3′, 5′-GCG ACC AAG TCC TTC CCA CTC GTG CAC ACT-3′, 409 bp; for transforming growth factor-α (TGF-α): 5′-ATG GTC CCC TCG GCT GGA CAG-3′, 5′-GGC CTG CTT CTT CTG GCT GGC-3′, 297 bp; for TGF-β1: 5′-GCC CTG GAC ACC AAC TAT TGC T-3′, 5′-AGG CTC CAA ATG TAG GGG CAG G-3′, 161 bp; and for TGF-β2: 5′-GAT TTC CAT CTA CAA GAC CAC GAG GGA CTT GC-3′, 5′-CAG CAT CAG TTA CAT CGA AGG AGA GCC ATT CG-3′, 503 bp. Each primer pair concentration versus 0.27 μM G3PDH primers was calibrated for each multiplex PCR reaction to detect unsaturated amplifications for both amplicons. These values were for Bax versus G3PDH, 0.3 μM; p21 versus G3PDH, 0.4 μM; for iNOS versus G3PDH, 0.3 μM; for c-jun versus G3PDH, 0.3 μM; for TGF-α versus G3PDH, 0.4 μM; for TGF-β1 versus G3PDH, 0.3 μM; and for TGF-β2 versus G3PDH, 0.4 μM. PCR products were electrophoresed in a 1% agarose gel with ethidium bromide staining and photographed under ultraviolet light.

Northern blot analysis

A 495-bp fragment of *CDKN1*^{p21} cDNA was amplified by RT-PCR (primers: forward 5′-ATGTCAGAACCGGCTGGGGATGTCCG-3′, reverse 5′-TTAGGGCTTCCTCTTGGAGAAGATCA-3′), subcloned into TA cloning vector (Invitrogen, Carlsbad, CA) and subsequently released with EcoR I and ^{32}P-labeled to make a probe. U251MG cells and LN382 cells were cultured for 48 hr at either 37° or 34°C, and RNA were extracted from the cells. RNA were electrophoresed on a formaldehyde/agarose gel and transferred to a nylon membrane (Hybond-N, Amersham, England). The membrane was

prehybridized in Hybrisol I (Oncor, Gaithersburg, MD), followed by hybridization with the *CDKN1*^{p21} probe at 42°C overnight. The membrane was washed and exposed to Kodak X-Omat AR film at −70°C for 4 –24 hr.

Cell growth count

Cells (5×10^4/dish) were plated in a 6-cm diameter Petri dish with 3 ml of culture medium and left overnight at 37°C to allow cells to adhere to the dish. The next morning the culture was continued at either 34° or 37°C (day 0). After day 1, duplicate dishes were trypsinized every day and cells were counted until day 9. For analysis of reversibility of growth arrest, cells (1×10^6/dish) were seeded and allowed to adhere overnight at 37°C, and then cultured (*i*) continuously at 37°C, (*ii*) at 34°C for 24 hr and then 37°C (day 0), (*iii*) at 34°C for 48 hr and then at 37°C (day 0) and (*iv*) continuously at 34°C. The numbers of the cells in triplicate dishes were counted daily until day 5.

Cell-cycle analysis

Cell cycle was analyzed by flow cytometry using a bromodeoxyuridine-propidium iodide (BrdU-PI) dual staining technique. Briefly, BrdU to a final concentration of 5 μg/ml was added to the cells (1×10^6) cultured at each condition in 6-cm dishes, and the cells were incubated at 37°C for 30 min. The cells were then harvested with trypsin-EDTA treatment, fixed with 70% ethanol, treated with 2 N HCl for 30 min and then neutralized with 0.1 M sodium tetraborate (pH 8.5). After washing in phosphate-buffered saline (PBS) containing 0.5% Tween 20 (Bio-Rad, Richmond, CA), the cells were incubated with a monoclonal anti-BrdU antibody (Becton-Dickinson, Lincoln Park, NJ) and then with a fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G (IgG) antibody (Tago, Burlingame, CA), respectively for 30 min at room temperature. After washing with PBS, the cells were incubated with 50 μg/ml PI for 30 min at 4°C in the dark. Thereafter, the sample was analyzed in a FACSCalibur cytometer (Becton-Dickinson); 5×10^3 cells were counted per sample.

p53 immunohistochemistry

LN382 or U251MG cells (5×10^4) were seeded on poly-L-lysine-coated glass slides in 2 ml medium (Becton Dickinson) and precultured at 37°C for 24 hr. The culture was subsequently continued for the next 24 hr at 34° or 37°C. The cells on the slides were fixed with acetone for 10 min and dried. The slides were treated with a mouse monoclonal anti-p53 protein antibody DO7 (Dako, Glostrup, Denmark) at a 200-fold dilution and left at 4°C overnight. The staining was revealed using a streptavidin-biotin complex method with a biotinylated anti-mouse IgG rabbit polyclonal antibody (Nichirei, Tokyo, Japan), and a Vectastain Elite ABC kit (Vector Japan, Tokyo). Diaminobenzidine was used as the chromogen. The nuclei were counterstained with hematoxylin.

p53 ELISA

ELISA for p53 protein was performed using Rapid Format Pan p53 ELISA kit (Calbiochem, San Diego, CA) according to the manufacturer's instructions. Protein extracted from duplicate wells of LN382 or U251MG cells 0, 12, 24, 48 or 72 hr after temperature switch from 37° to 34°C were quantified for p53 content. The p53 concentration (in nanograms) was expressed per milligrams total cellular protein, which was determined by the use of the BCA protein assay reagent (Pierce, Rockford, IL) and optical density measurement at 550 nm.

Adenoviral wild-type *TP53* gene transfer

To overexpress WT p53 in glioma cells we infected the glioma cells with an adenoviral vector expressing p53 as described previously. [15] Monolayer cultures of LN382 and U251MG cells were infected at a multiplicity of infection (m.o.i.) of 100. Adenoviruses expressing luciferase

(Ad-Luc) or β-galactosidase (Ad-LacZ) were used as a controls. Assay for sub-G_0/G_1 (apoptotic) cells with flow cytometry (PI staining) was performed 24 hr after the infection.

Generation of a clone expressing stably exogenous coxsackievirus and adenovirus receptor (CAR) in LN382 cells

Plasmid expressing human CAR cDNA[16] was stably transfected into LN382 cells by CLONfectin (Clontech, Palo Alto, CA). After 400 μg/ml of G418 selection for 2 weeks, individual clones were isolated, expanded and subjected to screening for efficiency of adenovirus infection and CAR expression. G418-positive transfectants were infected with 100 m.o.i. of Ad-LacZ virus for 72 hr followed by X-gal and the clones with highest numbers of blue cells were retained. Subsequently, immunostaining with an anti-CAR antibody (a gift from Dr. Jeffrey Bergelson) was performed and the clone with highest constitutive and homogeneous CAR expression was selected and designated LN382/CAR.

Ad-LacZ infection and X-gal staining

Cells of 1×10^5/well in 6-well tissue-culture plates were infected with 100 moi of Ad-LacZ for 72 hr. Cells were then washed twice with 5 ml cold PBS before fixing with ice cold 0.5% glutaraldehyde in PBS for 5 min at room temperature. Solution was aspirated and cells were washed twice with cold PBS before adding 2 ml of X-gal staining solution. After incubation at 37°C for 24 hr, cells were evaluated for the presence of β-galactosidase-expressing cells (blue). The X-gal staining solution contained the following reagents: 1.3 mM $MgCl_2$, 15 mM NaCl, 44 mM Tris, pH 7.4, 3 mM$K_4Fe(CN)_6$-$3H_2O$, 3 mM$K_3Fe(CN)_6$ and 0.5 mg/ml of X-gal.

Western blot to detect adenovirally transduced WT p53 protein

LN382 and LN382/CAR cells after 100 moi of Ad-p53 or Ad-LacZ infection for 72 hr at both 34°C and 37°C were harvested and lysed in 1× RIPA buffer (150 mM, 1.0% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris pH 8.0) containing 1× of Complete protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN) in ice for 10 min. Cellular extracts (30 μg) from each sample were subjected to Western blot analysis. After resolving on a 10% SDS-PAGE, proteins were transferred to a Hybond ECL nitrocellulose membrane (Amersham, Piscataway, NJ). The membrane was then hybridized with both mouse monoclonal anti-p53 protein antibody DO-1 (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal anti-actin antibody (Sigma, St. Louis, MO).

Three-dimensional modeling of Leu197 mutant p53

Three-dimensional (3D) modeling of Leu197 mutant p53 was done using a program Sybil (Tripos Inc.), applying crystallographic data by Cho *et al.*[17] The χ_1 and χ_2 angles of the Leu at position 197 were rotated manually to find the best conformation to fit the space.

RESULTS

Transcriptional activity of the LN382-derived p53 Leu197 mutant in yeast

Glioblastoma cell lines LN382 and U251MG (mutant p53 at amino acid 273, CGG → CAG; Arg → His) were tested for p53 transcriptional activity in yeast. U251MG cells gave 100% red colonies, indicating complete inactivation of p53 transcriptional activity of the His273 mutant present in this cell line. LN382 cells gave 100% white colonies with a slight pinkish haze at 30°C. Sequencing analyses of the p53 cDNA extracted from the yeast colonies demonstrated that LN382 had a missense mutation at codon 197 (GTG → CTG; Val → Leu) (Fig. 1*a*). All colonies had the same mutation, suggesting homozygosity. The slight pinkish haze present on colonies harboring the Leu197 mutant form of p53 suggested that it might be

a ts mutant with slightly decreased WT transcriptional activity at 30°C. To test the effect of temperature on the transcriptional activity of Leu197 mutant, the yeast colonies were cultured at 30°, 34° and 37°C. The colony color was white at 30°C, pink at 34°C, and red at 37°C (Fig. 1*b–d*), which indicates that transcriptional activity of Leu197 mutant is abrogated at the higher temperature. Color of colonies containing the His273 mutant derived from U251MG cells showed no significant change at any temperature (data not shown).

Transcriptional activity of p53 Leu197 mutant in LN382 glioblastoma cells

To examine whether the temperature modulation of transcriptional activity of the p53 Leu197 mutant observed in yeast is reflected in actual modification of gene expression in LN382 cells, we performed multiplex RT-PCR on total RNA extracted from LN382 cells grown at 32°, 34° and 37°C for 24 or 48 hr. mRNA levels were assessed by RT-PCR for genes previously reported to be upregulated [*Bax*, *CDKN1*^{p21}, transforming growth factor-α (*TGF*-α), *TGF*-β] or downregulated (*c-fos*, *c-jun*, *iNOS*) by p53.[18–23] Expression of *CDKN1*^{p21} and *TGF*-α was markedly increased at 34° and 32°C. *Bax* gene was expressed at a low level at 37°C and its expression was moderately increased at lower temperatures (Fig. 2*a*). Except for a slight reduction in *iNOS* gene expression 48 hr after temperature change to 32°C, expression of the other genes did not change significantly at the different temperatures. Induction of *CDKN1*^{p21} mRNA expression was further confirmed by Northern blot analysis. *CDKN1*^{p21} mRNA was induced in LN382 when the cells were cultured for 48 hr at 34°C, whereas the induction was not observed in U251MG cells (Fig. 2*b*). These results demonstrate that a 3°C temperature drop restores WT transcription activity to the p53 Leu197 mutant in human cells and that this activity is not augmented by further temperature decrease to 32°C, in contrast to what occurred in yeast cells.

p53 protein turnover is temperature dependent in LN382 cells

To test the effect of temperature on p53 Leu197 protein turnover, we measured the amount of p53 protein in the cells by immunocytochemistry and ELISA at 34° and 37°C. At 37°C, p53 immunocytochemistry showed an accumulation of p53 protein in cell nuclei of LN382 similar to that in U251MG cells used as positive controls. After the temperature switch to 34°C for 48 hr, immunoreactivity of p53 protein was markedly decreased in LN382 but not in U251MG, indicating a decline in p53 levels in LN382 cells at 34°C (Fig. 2*c–f*). ELISA also showed a significant decrease in p53 protein levels 12–72 hr after switch from 37° to 34°C in LN382 cells, whereas no significant change was observed in U251MG cells (Fig. 2*g*). These results show that at 37°C p53 Leu197 mutant accumulate to a level similar to that of mutant His273, whereas at 34°C it is markedly reduced, suggesting normalization of p53 turnover.

Effects of restoration of WT p53 function on cell growth in LN382 cells

To measure the effects of temperature on cell growth, we cultured LN382 and U251MG cells at 37° and 34°C, and counted the cell numbers as described in Materials and Methods. At 34° C, cell growth of LN382 cells was markedly retarded as compared to 37°C, whereas no significant change was observed in U251MG cells (Fig. 3*a*).

To examine the cause of growth inhibition in LN382 cells at 34°C, we analyzed the effect of temperature on cell-cycle progression by flow cytometry using cells treated with BrdU and PI. This method separates S-phase cells based on BrdU incorporation (vertical axis) and G_0/G_1 from G_2/M cells according to their DNA content measured through PI incorporation (horizontal axis). LN382 and U251MG cells were analyzed 6, 12, 18, 24 and 48 hr after the temperature switch to 34°C. Within 6 hr the number of LN382 cells in the G_0/G_1 phase increased and those in the S phase decreased (Fig. 3*b*, left). In concert with the G_0/G_1 increase there was also an accumulation of cells in G_2/M that culminated at 24 hr. This increase subsided at 48 hr, paralleled by an increase in S-phase entry. There was no significant difference in the cell-cycle

phase distribution of U251MG cells in the temperature switch from 37° to 34°C for 48 hr (Fig. 3*b*, right).

To examine whether the growth arrest was permanent, we incubated LN382 cells for 24 or 48 hr at 34°C and subsequently returned them to 37°C. As a result, we found that the cell growth arrest was reversible upon restoration of the nonpermissive temperature (Fig. 3*c*).

Temperature switch does not induce apoptosis in LN382 cells

To determine whether reduction of cellular growth was caused by induction of apoptosis in LN382 cells at 34°C, we examined DNA fragmentation in LN382 cells and U251MG cells cultured at 34° and 37°C. Both cell lines did not show any DNA fragmentation at either temperature (data not shown). Furthermore, no increase of sub-G_0/G_1 fraction indicative of apoptosis was observed in flow cytometry (data not shown).

High level expression of WT p53 induces apoptosis in LN382 cells

To delineate the reasons for absence of apoptosis induction in LN382 cells, that is, insufficient expression or defect(s) in p53-dependent apoptosis pathways in the cells, we transduced WT p53 with adenoviral gene transfer. By simple infection of p53 adenovirus, LN382 cells did not undergo apoptosis as assessed by flow-cytometric analysis, whereas massive apoptosis occurred in U251MG cells (Fig. 4*a*).

Because adenoviral gene transduction is dependent on cellular expression of CAR,[24] we tested adenoviral infectivity on LN382 cells using adenovirus carrying the β-*gal* gene. Adenoviral transduction efficiency was very low both at 37°C and 34°C (Fig. 4*b*, upper panel). We therefore transfected the *CAR* gene into LN382 cells and selected for a stable clone expressing CAR and infecting at high efficiency with adenovirus (LN382/CAR) (see Material and Methods). Increased CAR expression was sufficient to significantly augment the efficiency of Ad-LacZ infection, and consequently *LacZ* gene transduction and the number of blue cells after X-gal staining (Fig. 4*b*, lower panel). In contrast to the cells without CAR transfection, the cells transfected with CAR underwent apoptosis at both 37° and 34°C by adenoviral WT p53 gene transfer (Fig. 4*c*). Furthermore, LN382/CAR cells infected with Ad-LacZ did not die at 34°C, suggesting that apoptosis is induced by increased p53 levels rather than a synergy of activation of endogenous p53 with cellular responses to viral infection. Western blot analysis confirmed a considerably higher level of p53 expression in LN382/CAR cells after adenoviral WT p53 transfer at both temperatures (Fig. 4*d*).

3D structure of Leu197 mutant

The effect of a Val to Leu mutation at codon 197 on the 3D structure of p53 protein was examined using a computer program. Substitution of Val to Leu creates an increased volume of the side chain: —CH—$(CH_3)_2$ to —CH_2—$CH(CH_3)_2$. The side chain bumped into Val16, Val216, Val218, Ile232 or Tyr234, according to the rotational angles (χ_1 and χ_2) of the side chain. A search for the best conformation disclosed that Leu197 side chain fits the space when $\chi_1 = 172°$ and $\chi_2 = 180°$. The closest amino acid is Val16 (distance 2.9 Å). Overall, the crystal structure indicated that the Val to Leu mutation at codon 197 does not directly interfere with the p53 binding to the DNA, but leads to a structural change that might result in a molecule with an unstable conformation.

DISCUSSION

In our study we have described a glioblastoma cell line containing an endogenous ts *TP53* mutant allele. Transcriptional activity of the Leu197 mutant was restored in the cells by lowering the culture temperature by only 3°C. With the restoration of WT p53 function, the

cells completely stopped growing mainly because of G_1-phase cell-cycle arrest. A slight transitional arrest was observed in G_2/M, which may have originated from p53's ability to inhibit the $p34^{cdc2}$/cyclin B1 complex.[25,26] This growth arrest was reversible upon temperature rise, indicating that the arrest was mediated by p53's temperature-dependent transcriptional activity.

Restoration of WT p53 function induced *Bax*, *CDKN1*^{p21}, and *TGF*-α, whose promoters contain p53-responsive elements, in agreement with previous reports.[18,22,23] It did not induce *TGF*-β1 or *TGF*-β2, which were reported to be induced by WT p53.[20] These two genes, however, are unlikely to be directly regulated by p53, because their promoters do not contain p53-responsive elements. *c-fos* and *iNOS* genes are known to be repressed by WT p53,19,[21] among which only a slight repression was observed for iNOS. It may be due to a limitation in sensitivity of multiplex RT-PCR assay, or a difference in the level of WT p53 expressed (close to physiologic levels in our present study and extremely high in previous reports using plasmid vectors).

The restoration of WT function did not induce apoptosis in LN382 cells. Because restoration of WT p53 function at 34°C induced the *Bax* gene in LN382 cells, this inability to cause apoptosis is unlikely to be attributable to incomplete or unbalanced transactivating potential of p53 Leu197 ts mutant. This cell line did not have a defect in the downstream apoptotic signal pathway because high levels of WT p53 expression obtained with adenoviral p53 transfer induced apoptosis in LN382 cells. This effect was not a result of a synergistic effect of adenovirus infection with endogenous p53 activation, for Ad-LacZ infection did not induce apoptosis at 34°C. The failure in inducing apoptosis by the restoration of endogenous p53 function at 34°C is thus most likely attributable to the relatively low level of p53 expression under conditions of normalized protein turnover (Fig. 2*c–g*). This leads us to an important insight about proper uses of 2 functions of p53—G_1 arrest and apoptosis. A low level expression of p53 causes G_1 arrest, whereas a high level expression causes apoptosis. The latter case might occur with decreased Mdm2-mediated p53 turnover after phosphorylation on Ser15 and Ser37,[27] or increased transcriptional activity on apoptosis-related gene promoters by site-specific phosphorylation.[28]

It should be noted that cold stress can induce certain effects on cellular metabolism, cell growth and cell death. Such cellular effects have been studied mostly with temperatures less than 20° C. Such severe hypothermia can change the lipid composition of cellular membranes, and suppresses the rate of protein synthesis and cell proliferation. Milder hypothermia at 32°C has been found to induce a cold shock protein, namely CIRP (cold-inducible RNA-binding protein), which plays an essential role in cold-induced suppression of cell proliferation.[29] Whereas CIRP might have contributed to the cellular growth suppression observed in LN382, it is unlikely to have caused growth arrest per se at 34°C, because the growth and cell cycle of U251MG cells were barely altered by this modest temperature shift (Fig. 3*a*,*b*).

Whereas various mutations at codon 197 have been identified in 14 cases of cancers in diverse organs,[30] only 1 case of lung squamous cell carcinoma has shown a Leu197 mutation[31] and no cell line was established. Intrinsic ts p53 mutants have been described in several other human cancer cell lines at codons Phe172, Arg214, Cys234, Met272 and Lys285,[32,33] but the almost complete restoration of WT function with a slight change of temperature in LN382 cells as in our study is unique.

Amino acid 197 is in the S5 β-sheet of the p53 protein next to the H1 loop, which participates in chelating a Zn atom.[17] By 3D-image analysis, we showed that substitution of valine (—CH —$(CH_3)_2$) to leucine (—CH_2—$CH(CH_3)_2$) caused bumping of the terminal apolar hydrocarbon (—CH_3) of the Leu197 side chain into a distance of less than 4.0 Å to —CH_3 of

either Val16, Val216, Val218, Ile232 or Tyr234, as demonstrated in the 3D-conformational study. Because the van der Waals radius of —CH_3 is 2.0 Å,[34] distances between hydrocarbons of less than 4.0 Å produce repulsive forces between side chains, resulting in slacking of the folding made by N-terminus and S5-, S6-, S7-, and S8-β sheets. This may or may not affect Zn binding. In addition, valine (—CH—$(CH_3)_2$) and leucine (—CH_2—$CH(CH_3)_2$) contribute differently to side chain– helix interactions and thermal stability.[35] It is, therefore, conceivable that the change of Val197 to Leu197 makes an exquisite reversible conformational change upon only a subtle temperature change. It is intriguing, in this regard, that the prolonged turnover of the protein in its mutant conformation was rapidly normalized at 34°C, as deduced from the disappearance of nuclear staining of the protein and the ELISA assay. This finding suggests that this conformational switch might affect MDM2 binding required for p53 protein degradation.[36,37]

In conclusion, we have demonstrated that restoration of endogenous WT p53 activity in glioblastoma cells arrests their growth in the G_0/G_1 phase of the cell cycle. This finding is in contrast to normal human astrocytes in which WT p53 activity does not prevent cell growth. These results suggest that an endogenous signal present in the cancer cell induces p53 or synergistically acts with p53 to induce growth arrest. Such a signal might be activation of an oncogene or DNA damage resulting from genetic instability because both signals are known to occur in cancer and activate p53-mediated growth arrest.[38–40] This endogenous signal is likely to precede and drive the selection for mutation of the *TP53* gene in these cells. This cell line will be invaluable to define such signals and for further investigations on the role of p53 in cellular responses to variable stimuli or damages, including chemo- and radiotherapies.

Acknowledgments

We thank Dr. K. Gernert (BIMCORE facility, Emory University) for help with the 3D modeling, Dr. J. Bergelson for providing CAR cDNA and anti-CAR antibodies, Dr. N. de Tribolet for the LN-382 cell line and Ms. Y. Shinohe and Ms. M. Sudo for technical assistance. Dr. E. G. Van Meir was supported by grants from the Swiss National Science Foundation (31-49194.96), the NIH (CA86335 and NS41403) and MBNA, N.A.

REFERENCES

1. Fulci G, Ishii N, Van Meir EG. p53 and brain tumors: from gene mutations to gene therapy. Brain Pathol 1998;8:599–613. [PubMed: 9804370]
2. Nozaki M, Tada M, Kobayashi H, Zhang CL, Sawamura Y, Abe H, et al. Roles of the functional loss of p53 and other genes in astrocytoma tumorigenesis and progression. Neurooncology 1999;1:149–162.
3. Tada M, Matsumoto R, Iggo RD, Onimaru R, Shirato H, Sawamura Y, et al. Selective sensitivity to radiation of cerebral glioblastomas harboring p53 mutation. Cancer Res 1998;58:1793–1797. [PubMed: 9581814]
4. Iwadate Y, Fujimoto S, Tagawa M, Namba H, Sueyoshi K, Hirose M, et al. Association of p53 gene mutation with decreased chemosensitivity in human malignant gliomas. Int J Cancer 1996;69:236–240. [PubMed: 8682593]
5. Weller M. Predicting response to cancer chemotherapy: the role of p53. Cell Tissue Res 1998;292:435–445. [PubMed: 9582400]
6. Mercer WE, Shields MT, Amin M, Sauve GJ, Appella E, Romano JW, et al. Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. Proc Natl Acad Sci U S A 1990;87:6166–6170. [PubMed: 2143581]
7. Van Meir EG, Polverini PJ, Chazin VR, Su Huang HJ, de Tribolet N, Cavenee WK. Release of an inhibitor of angiogenesis upon induction of wild type p53 expression in glioblastoma cells. Nat Genet 1994;8:171–176. [PubMed: 7531056]
8. Trepel M, Groscurth P, Malipiero U, Gulbins E, Dichgans J, Weller M. Chemosensitivity of human malignant glioma: modulation by p53 gene transfer. J Neurooncol 1998;39:19–32. [PubMed: 9760067]

9. Van Meir EG, Roemer K, Diserens AC, Kikuchi T, Rempel SA, Haas M, et al. Single cell monitoring of growth arrest and morphological changes induced by transfer of wild-type p53 alleles to glioblastoma cells. Proc Natl Acad Sci U S A 1995;92:1008–1012. [PubMed: 7862624]
10. Gjerset RA, Turla ST, Sobol RE, Scalise JJ, Mercola D, Collins H, et al. Use of wild-type p53 to achieve complete treatment sensitization of tumor cells expressing endogenous mutant p53. Mol Carcinog 1995;14:275–285. [PubMed: 8519417]
11. Ishii N, Maier D, Merlo A, Tada M, Sawamura Y, Diserens AC, et al. Frequent co-alterations of TP53, p16/CDKN2A, p14ARF, PTEN tumor suppressor genes in human glioma cell lines. Brain Pathol 1999;9:469–479. [PubMed: 10416987]
12. Estreicher A, Iggo R. Retrovirus-mediated p53 gene therapy. Nat Med 1996;2:1163. [PubMed: 8898732]
13. Flaman JM, Frebourg T, Moreau V, Charbonnier F, Martin C, Chappuis P, et al. A simple p53 functional assay for screening cell lines, blood, and tumors. Proc Natl Acad Sci U S A 1995;92:3963–3967. [PubMed: 7732013]
14. Tada M, Iggo RD, Ishii N, Shinohe Y, Sakuma S, Estreicher A, et al. Clonality and stability of the p53 gene in human astrocytic tumor cells: quantitative analysis of p53 gene mutations by yeast functional assay. Int J Cancer 1996;67:447–450. [PubMed: 8707423]
15. Tada M, Sakuma S, Iggo RD, Saya H, Sawamura Y, Fujiwara T, et al. Monitoring adenoviral p53 transduction efficiency by yeast functional assay. Gene Ther 1998;5:339–344. [PubMed: 9614553]
16. Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS, et al. Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. Science 1997;275:1320–1323. [PubMed: 9036860]
17. Cho Y, Gorina S, Jeffrey PD, Pavletich NP. Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. Science 1994;265:346–355. [PubMed: 8023157]
18. el Deiry WS, Harper JW, O'Connor PM, Velculescu VE, Canman CE, Jackman J, et al. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res 1994;54:1169–1174. [PubMed: 8118801]
19. Forrester K, Ambs S, Lupold SE, Kapust RB, Spillare EA, Weinberg WC, et al. Nitric oxide-induced p53 accumulation and regulation of inducible nitric oxide synthase expression by wild-type p53. Proc Natl Acad Sci U S A 1996;93:2442–2447. [PubMed: 8637893]
20. Fujiwara T, Mukhopadhyay T, Cai DW, Morris DK, Roth JA, Grimm EA. Retroviral-mediated transduction of p53 gene increases TGF-β expression in a human glioblastoma cell line. Int J Cancer 1994;56:834–839. [PubMed: 8119773]
21. Ginsberg D, Mechta F, Yaniv M, Oren M. Wild-type p53 can down-modulate the activity of various promoters. Proc Natl Acad Sci U S A 1991;88:9979–9983. [PubMed: 1946467]
22. Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 1995;80:293–299. [PubMed: 7834749]
23. Shin TH, Paterson AJ, Kudlow JE. p53 stimulates transcription from the human transforming growth factor α promoter: a potential growth-stimulatory role for p53. Mol Cell Biol 1995;15:4694–4701. [PubMed: 7651386]
24. Asaoka K, Tada M, Sawamura Y, Ikeda J, Abe H. Dependence of efficient adenoviral gene delivery in malignant glioma cells on the expression levels of the coxsackievirus and adenovirus receptor. J Neurosurg 2000;92:1002–1008. [PubMed: 10839262]
25. Innocente SA, Abrahamson JL, Cogswell JP, Lee JM. p53 regulates a G2 checkpoint through cyclin B1. Proc Natl Acad Sci U S A 1999;96:2147–2152. [PubMed: 10051609]
26. Zhan Q, Antinore MJ, Wang XW, Carrier F, Smith ML, Harris CC, et al. Association with Cdc2 and inhibition of Cdc2/Cyclin B1 kinase activity by the p53-regulated protein Gadd45. Oncogene 1999;18:2892–2900. [PubMed: 10362260]
27. Shieh SY, Ikeda M, Taya Y, Prives C. DNA damage-induced phosphorylation of p53 alleviates inhibition by mdm2. Cell 1997;91:325–334. [PubMed: 9363941]
28. Oda K, Arakawa H, Tanaka T, Matsuda K, Tanikawa C, Mori T, et al. p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. Cell 2000;102:849–862. [PubMed: 11030628]

29. Fujita J. Cold shock response in mammalian cells. J Mol Microbiol Biotechnol 1999;1:243–255. [PubMed: 10943555]
30. Hainaut P, Hernandez T, Robinson A, Rodriguez Tome P, Flores T, Hollstein M, et al. IARC Database of p53 gene mutations in human tumors and cell lines: updated compilation, revised formats and new visualisation tools. Nucleic Acids Res 1998;26:205–213. [PubMed: 9399837]
31. Vahakangas KH, Samet JM, Metcalf RA, Welsh JA, Bennett WP, Lane DP, et al. Mutations of p53 and ras genes in radon-associated lung cancer from uranium miners. Lancet 1992;339:576–580. [PubMed: 1347094]
32. Jia LQ, Osada M, Ishioka C, Gamo M, Ikawa S, Suzuki T, et al. Screening the p53 status of human cell lines using a yeast functional assay. Mol Carcinog 1997;19:243–253. [PubMed: 9290701]
33. Siddik ZH, Mims B, Lozano G, Thai G. Independent pathways of p53 induction by cisplatin and X-rays in a cisplatin-resistant ovarian tumor cell line. Cancer Res 1998;58:698–703. [PubMed: 9485023]
34. Tsai J, Taylor R, Chothia C, Gerstein M. The packing density in proteins: standard radii and volumes. J Mol Biol 1999;290:253–266. [PubMed: 10388571]
35. Hermans J, Anderson AG, Yun RH. Differential helix propensity of small apolar side chains studied by molecular dynamics simulations. Biochemistry 1992;31:5646–5653. [PubMed: 1610812]
36. Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. Nature 1997;387:296–299. [PubMed: 9153395]
37. Kubbutat MH, Jones SN, Vousden KH. Regulation of p53 stability by Mdm2. Nature 1997;387:299–303. [PubMed: 9153396]
38. Barlow C, Brown KD, Deng CX, Tagle DA, Wynshaw-Boris A. Atm selectively regulates distinct p53-dependent cell-cycle checkpoint and apoptotic pathways. Nat Genet 1997;17:453–456. [PubMed: 9398849]
39. Goga A, Liu X, Hambuch TM, Senechal K, Major E, Berk AJ, et al. p53 dependent growth suppression by the c-Abl nuclear tyrosine kinase. Oncogene 1995;11:791–799. [PubMed: 7651743]
40. Zhang Y, Xiong Y, Yarbrough WG. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. Cell 1998;92:725–734. [PubMed: 9529249]

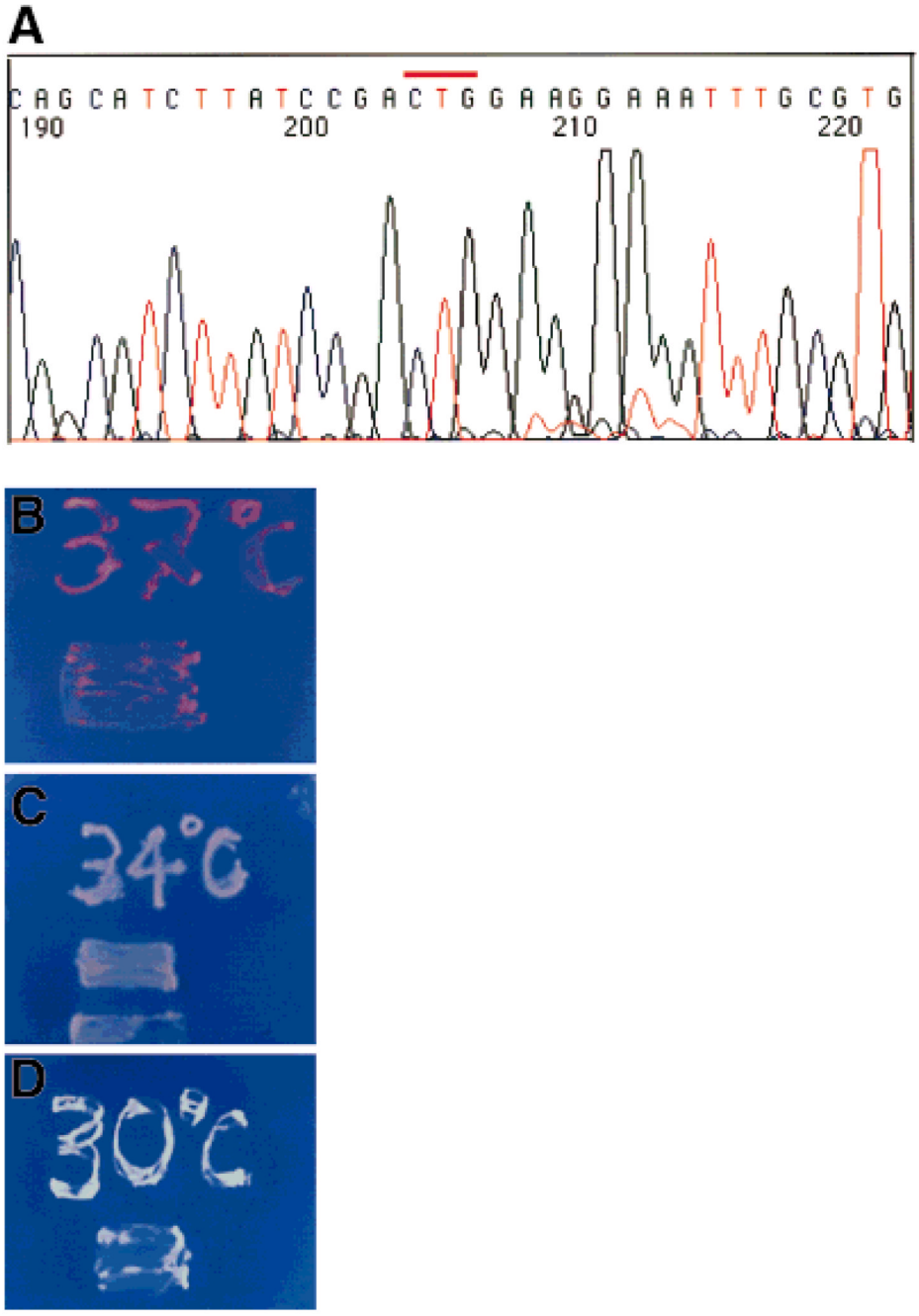

FIGURE 1.

Sequence of the 197Leu p53 mutant and temperature sensitivity of its transcriptional activity in yeast cells. (*a*) Sequence chromogram showing a missense mutation [(GTG (Val) → CTG (Leu), red bar] at codon 197 of the p53 cDNA extracted from yeast colonies that originated from reverse-transcribed RNA of LN382 human glioblastoma cells. (*b*)–(*d*) A yeast colony containing the 197Leu p53 mutant was replated and cultured at different temperatures. When expressed in yeast, p53 protein can transactivate the exogenous p53-responsive 3×RGC-CYC promoter driving the *ADE2* gene. This rescues adenine deficiency in the ADE^{-} yeast strain yIG397 and results in the formation of white colonies. At 37°C (*b*), the yeast color is red,

indicating the absence of p53 transcriptional activity. At 34°C (*c*), pink color shows a partial transcriptional activity. At 30°C (*d*), white color indicates a high transcriptional activity of p53.

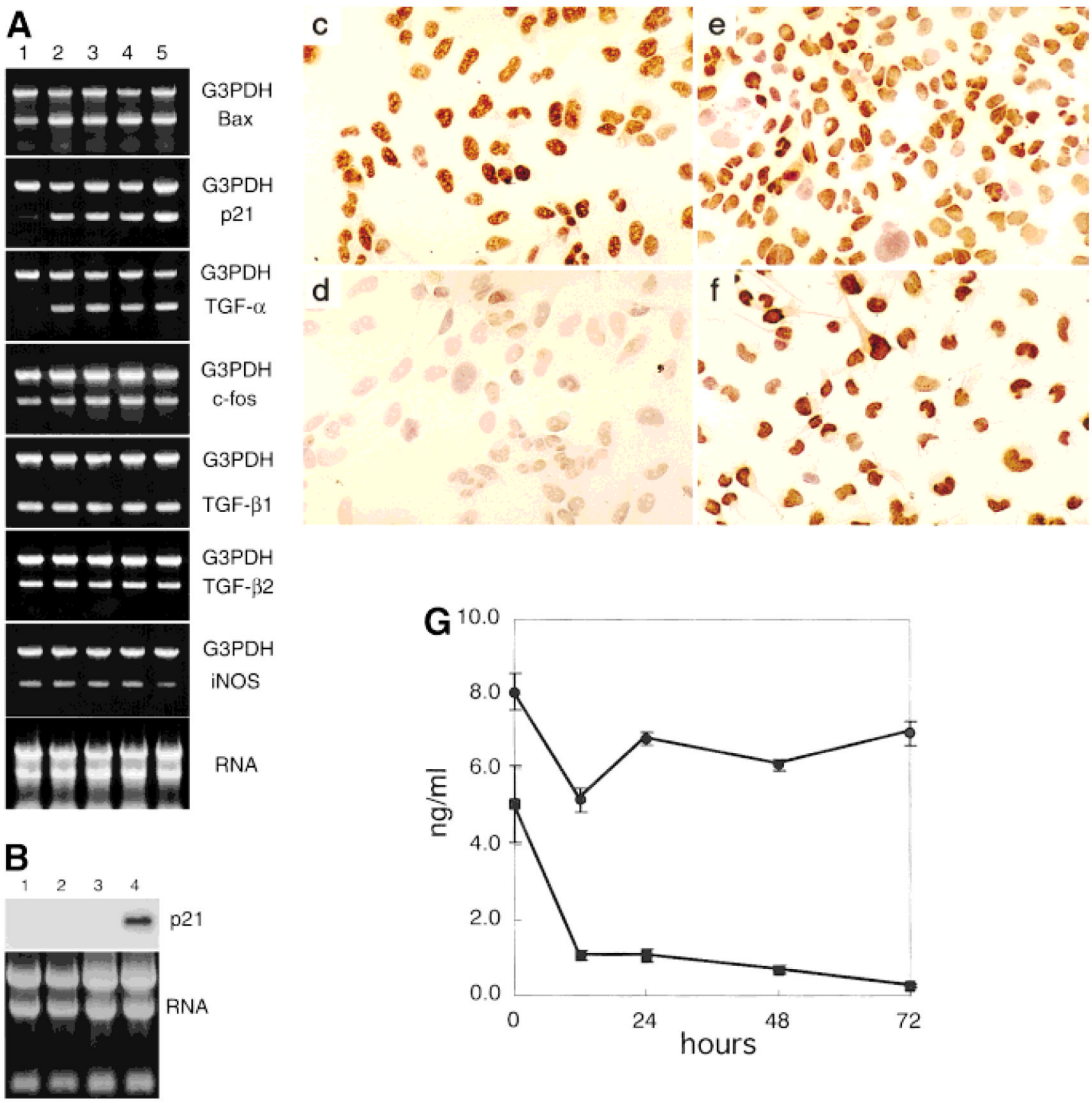

FIGURE 2.

Temperature sensitivity of the p53 Leu197 mutant transcriptional activity in LN382 human glioblastoma cells. (*a*) Multiplex reverse transcription-polymerase chain reaction for gene expression in LN382 cells cultured at different temperatures. G3PDH (glyceraldehyde 3-phosphate dehydrogenase) was used as an internal control. Lane 1, 37°C; lane 2, 24 hr after culture at 34°C; lane 3, 48 hr after culture at 34°C; lane 4, 24 hr after culture at 32°C; lane 5, 48 hr after culture at 32°C. The lowest panel shows 18S and 28S rRNA to demonstrate equal RNA loading of the samples and RNA integrity. (*b*) Northern blot analysis for mRNA expression of the *CDKN1*^{p21} gene. Lane 1, U251MG cells cultured at 37°C; lane 2, U251 cells at 34°C; lane 3, LN382 cells at 37°C; lane 4, LN382 cells at 34°C. (*c*)–(*f*) Immunocytochemistry for p53 using DO7 antibody. Nuclear accumulation of p53 protein is observed in LN382 cultured at 37°C and U251MG cells cultured at 34° and 37°C for 48 hr, but not in LN382 cells cultured at 34°C. (*c*) LN382, 37°C; (*d*) LN382, 34°C; (*e*) U251MG, 37°C; (*f*) U251MG, 34° C. (*g*) Chronological measurement of p53 protein contents by enzyme-linked immunosorbent assay in U251MG cells (●) and in LN382 cells (■) 12, 24, 48 and 72 hr after switch from 37° to 34°C.

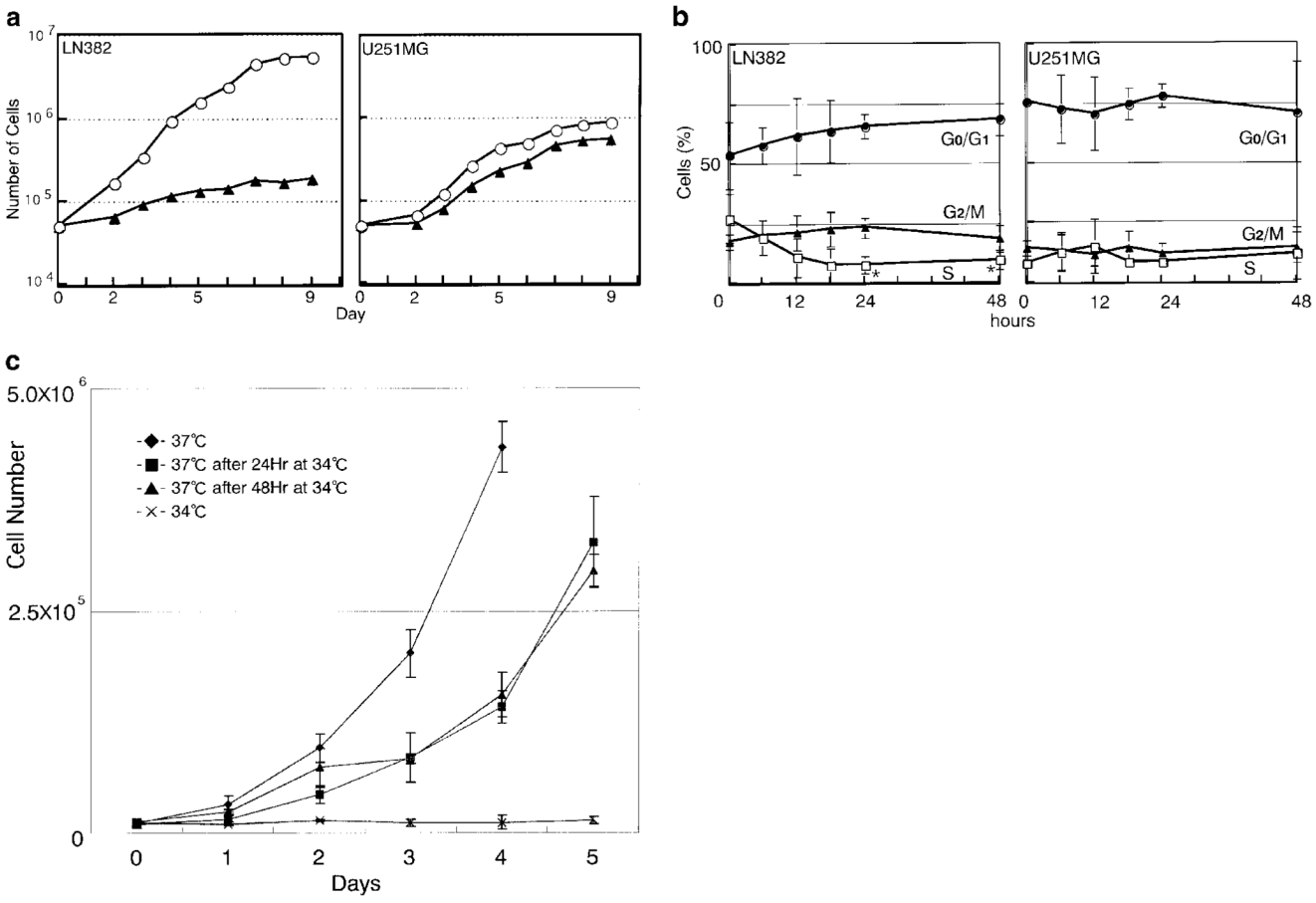

FIGURE 3.

Cell growth kinetics and cell cycle progression analyses of LN382 and U251MG cells cultured at different temperatures. (*a*) LN382 (left) and U251MG (right) cells were cultured at 37°C (○) or 34°C (▲) and counted every day for 9 days in duplicate wells (*b*). The temperature was switched from 37° to 34°C, and distribution of cells in respective cell cycle phases was measured at 6, 12, 18, 24 and 48 hr by dual flow cytometry for BrdU and PI. Percentages of cells in the G_0/G_1, G_2/M and S phases of the cell cycle are plotted separately. The assay was performed in triplicate dishes and standard deviations are shown. *Statistically significant difference compared with 0 hr ($p < 0.05$, *t*-test). (*c*) Reversal of cell growth arrest in LN382 cells. Cells were pre-cultured for 24 or 48 hr at 34°C, and culture was continued at 34° or 37° C from day 0. For control, cells were cultured continuously at 34° or 37°C from day 0. The number of the cells in triplicate dishes were counted every day until day 5.

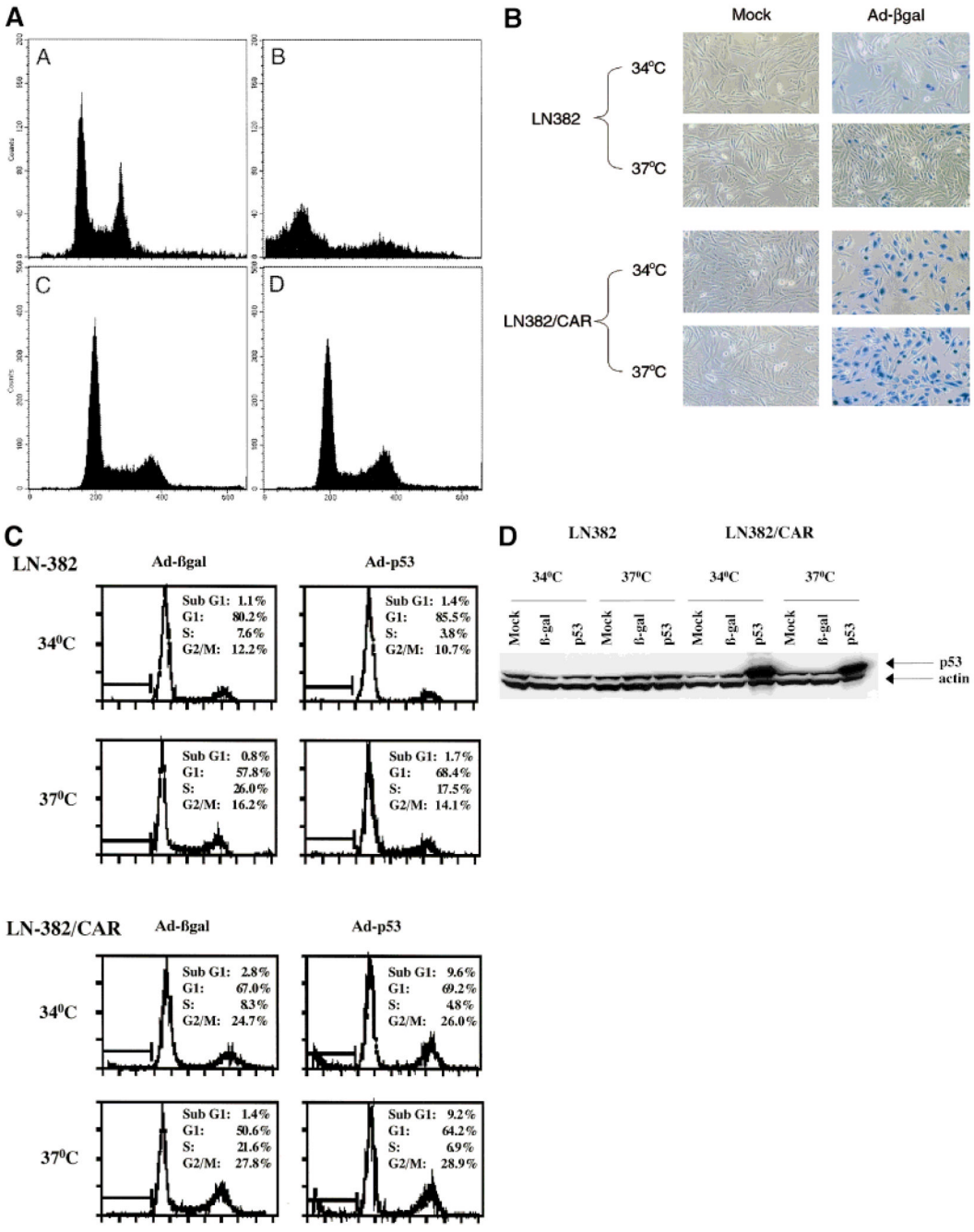

FIGURE 4.

(*a*) Induction of apoptosis by adenoviral wild-type p53 transfer in U251MG cells but not in LN382 cells. U251MG cells (A and B) and LN382 cells (C and D) were infected with a multiplicity of infection of 100 of adenovirus for high level transduction of wild-type p53 (B and D) or control luciferase genes (A and C). Cells were examined with flow cytometry to detect sub-G_0/G_1 apoptotic cells. (*b*) Increased adenoviral infectivity by forced expression of coxsackievirus and adenovirus receptor (CAR) in LN382 cells. LN382 cells without CAR transfection showed low levels of β-gal transduction (upper panels), but LN382 cells with CAR transfection (lower panels) showed increased transduction efficiencies both at 34° and 37°C. (Mock, LN382 cells infected with control empty vector.) (*c*) Induction of apoptosis in LN382

cells by CAR+ adenoviral p53 transduction. LN382 cells without CAR transfection before adenoviral β-gal or p53 transduction did not show apoptosis as sub-G_1 cells (upper panels), but cells with CAR plus p53 transduction underwent apoptosis (lower panels). (*d*) Detection of a high-level wild-type p53 protein in LN382 cells transfected with CAR and adenoviral p53. LN382 cells with or without CAR transfection were infected with control empty vector (Mock), Ad-β-gal vector, or Ad-p53 vector. Cell lysates were electrophoresed in an sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nylon membrane, and p53 protein was detected with a monoclonal antibody.

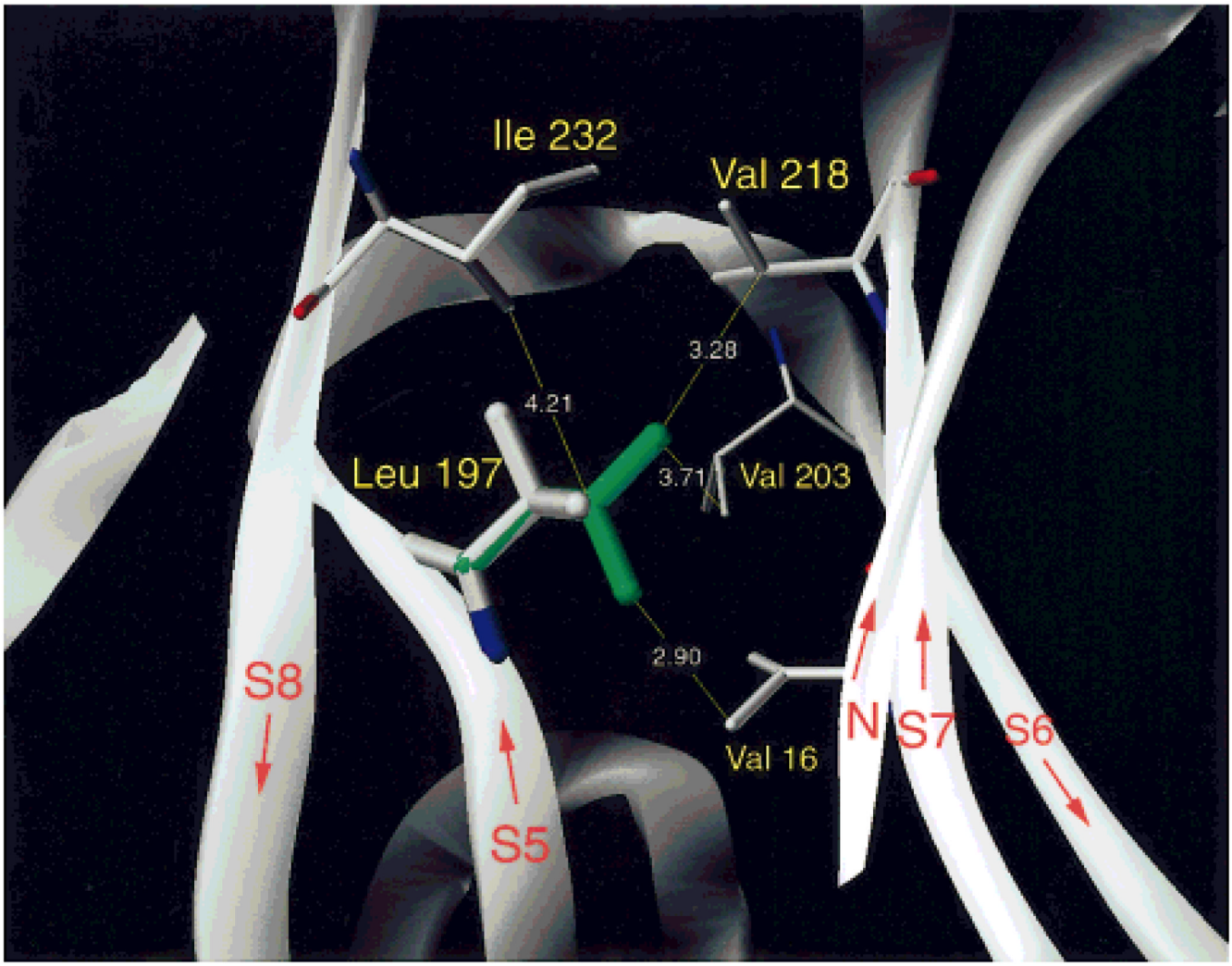

FIGURE 5.

Local 3-dimensional structure of the mutant p53 protein around amino acid 197. The normal structure in gray and the mutant amino-acid (Leu) side chain is superimposed in green. Distances to the side chains of close amino acids are shown in Angstroms, at the best-fit angles (χ_1 = 172°, χ_2 = 180°) of the Leu side chain. Volume increase in the side chain is considered to produce slacking of the folding made by N-terminus and S5-, S6-, S7- and S8-beta sheets, leading to an unstable conformation.