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A novel p53-inducible gene coding for a microtubule-localized protein with G_2-phase-specific expression

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Wild-type (wt) p53 can act as a sequence-specific transcriptional activator and it is believed that p53 elicits at least part of its biological effects by regulating the expression of specific target genes. By using a differential subtractive hybridization approach in a murine cell line stably transfected with a temperature-sensitive p53 mutant (Val135), we isolated a set of genes markedly induced by wt p53. One of them, provisionally named B99, was further characterized; its transcriptional induction was dependent on wt p53 function and the corresponding protein product was shown to accumulate after DNA damage in different cell types. Immunofluorescence analysis located the B99 protein to the microtubule network. Flow cytometry revealed that upon activation of p53 function the endogenous B99 protein was selectively induced in the G_2 fraction of the cell population. When B99 was ectopically expressed in p53-null murine fibroblasts, B99-transfected cells displayed an increased fraction with a 4N DNA content, indicative of interference with G_2 phase progression. Taken together these data suggest that B99 might play a role in mediating specific biological activities of wt p53 during the G_2 phase.

Keywords: G_2-specific/microtubule-localized protein/p53/p53-induced gene/Val5 fibroblasts

Introduction

One of the most frequent genetic events associated with the development of human cancer is the inactivation or loss of the normal function of the tumour suppressor gene p53 (Hollstein *et al*., 1991). A large body of evidence has accumulated suggesting wild-type (wt) p53 (but not its mutant derivatives) as a mediator of multiple antiproliferative activities including cell-cycle arrest, induction of apoptotic cell death and suppression of oncogene-mediated transformation (for review see Bates and Vousden, 1996; Ko and Prives, 1996; Hansen and Oren, 1997; Levine, 1997). The biochemical mechanisms by which wt p53 can elicit all of these activities have not been completely defined. However, an essential aspect of p53 function depends on it being a transcription factor. In fact, p53 contains a strong transcriptional activation domain within its N-terminus, it binds DNA with a well-defined cognate binding sequence and activates transcription of genes carrying such consensus sites in their promoter sequences (Lin *et al*., 1994; Levine, 1997). Apart from its function as a transcriptional activator, p53 can also repress the transcription of several genes (Sabbatini *et al*., 1995; Murphy *et al*., 1996 and references therein) and important aspects of its biological functions might be mediated through direct interaction with specific protein partners (Ko and Prives, 1996; Levine, 1997 and references therein).

Much evidence indicates that p53 acts primarily by arresting cells at the G_1/S restriction point and this effect has been clearly correlated to induction of Waf-1, a potent inhibitor of G_1-specific cyclin-dependent kinases (El-Deiry *et al*., 1993; Xiong *et al*., 1993; Waldman *et al*., 1996). Nevertheless, studies conducted on cells derived from p21Waf1–/– mice showed that loss of Waf-1 only partially abolishes the G_1 arrest function associated with wt p53 (Brugarolas *et al*., 1995; Deng *et al*., 1995). So this is probably mediated by multiple pathways in which other p53-regulated genes might play a role (Levine, 1997).

It has also been suggested that wt p53 plays a role in control of the G_2 phase. p53 has been shown to interact with the centrosomes (Brown *et al*., 1994) and p53-null cells frequently develop an aberrant number of centrosomes (Fukasawa *et al*., 1996). Fibroblasts from p53–/– mice are deficient in the checkpoint that blocks cell-cycle and prevents S phase entry when cells are treated with spindle-inhibitory drugs such as nocodazole (Cross *et al*., 1995). Furthermore, in several cell lines carrying an inducible p53 allele, it has been shown that p53 activation can arrest at both G_1/S and G_2/M phases of the cell cycle (Agarwal *et al*., 1995; Stewart *et al*., 1995). While the importance of p21Waf1 in G_1 arrest is well established, the role of p21Waf1 in such G_2-specific functions is still unclear (Deng *et al*., 1995; Lanni and Jacks, 1998) and it is reasonable to hypothesize that other p53-target genes could be involved. A recent report showed that wt p53 specifically upregulates the σ member of the 14-3-3 protein family, and that 14-3-3σ is capable of inducing G_2/M cell-cycle arrest, possibly by targeting cdc25c phosphatase (Furnari *et al*., 1997; Hermeking *et al*., 1997). It is also worth noting that p53 negatively regulates the expression of MAP4, a microtubule stabilizing protein whose intracellular relocalization has been correlated to p53-dependent apoptosis (Olmsted, 1991; Murphy *et al*., 1996). This links p53 to microtubule dynamics and cytoskeletal functions, but also suggests a possible correlation to G_2-

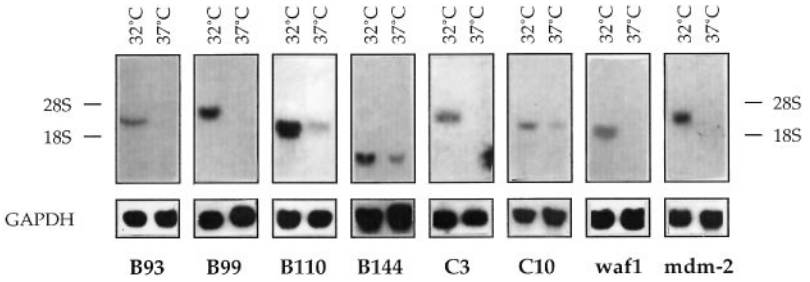

Fig. 1. Northern blot analysis of cDNA clones isolated during the screening. Total RNA was prepared from Val5 cells cultured at either 32 or 37°C. Lanes labelled 32°C contain a 1:1 mixture of RNA extracted 12 and 24 h after temperature down shift. 10 μg of RNA were loaded on each lane and hybridizations were performed under high stringency conditions. Exposure times varied between overnight and 7 days at –80°C with intensifying screens. Blots were also probed for GAPDH as a control.

specific cell-cycle events; in fact, MAP4 is phosphorylated by cdc2/B kinase at the G_2/M transition (Ookata *et al*., 1995), and progression of the cell cycle through G_2/M is linked to the status of microtubules (Andreassen and Margolis, 1994).

To further characterize p53 functions with respect to cell-cycle control, we screened for p53 target genes in a cellular system in which regulated induction of wt p53 causes an efficient and reversible growth arrest with no significant evidence of apoptosis. In this study we report the cloning of six cDNAs to be considered as potential p53-responsive genes. We focus on one of these, provisionally named B99, and provide evidence that it is a direct target for transcriptional activation by p53. Clone B99 encodes a novel protein that is localized to microtubules. When we characterized the p53-dependent regulation of B99, we found that induction of B99 protein was restricted to the G_2 population of cells, providing a notable example of a p53 target gene with cell-cycle-dependent expression. Ectopic expression of clone B99 reduced cell growth and caused a delay at the G_2/M phase of the cell cycle, as determined by flow cytometry. This evidence suggests that B99 could be involved in mediating the G_2-specific biological activities of wt p53.

Results

Isolation of novel p53-regulated genes by subtractive hybridization

In order to identify novel transcripts regulated upon induction of biochemically active p53, we used a subtractive hybridization approach in Val5 cells (Wu and Levine, 1994). The Val5 cell line is derived from p53-deficient Balb/c (10)1 mouse fibroblasts (Harvey and Levine, 1991) by stable transfection of the temperature-sensitive Val135 allele of murine p53 (Michalovitz *et al*., 1990; Martinez *et al*., 1991); at the permissive temperature of 32°C these cells upregulate p53 transcriptional targets like waf-1 or mdm-2 (Figure 1) and undergo an efficient and reversible G_1 arrest (Del Sal *et al*., 1996).

Two subtracted cDNA probes, one representing mRNAs expressed at the permissive temperature (target probe) and the other representing mRNAs expressed at the restrictive temperature (driver probe), were used in a differential-subtractive screening of a cDNA library constructed from mRNA of Val5 cells grown at 32°C. Enrichment and specificity of target and driver probes were evaluated by slot-blot analysis on a panel of sample genes, before and after subtractive hybridization. Transcripts abundantly expressed, such as GAPDH and 28S RNA, were significantly subtracted; in contrast, known p53-induced genes such as waf-1 and mdm-2, were significantly enriched in the subtracted target probe (data not shown). More than 500 plaques corresponding to differential signals were picked from the primary screening and analysed in a secondary screening by Southern blots. Polyclonal plaques corresponding to primary signals were excised *in vivo*, digested at the cloning sites, run in duplicate on the same agarose gels and blotted. Each of the duplicate blots was hybridized either with the target or the driver probes used for the primary screening. DNA fragments corresponding to differential signals were excised from agarose gels and used as probes to evaluate the expression of the corresponding transcripts in Val5 cells at 37 and 32°C. During the screening several clones corresponding to waf-1 and mdm-2 were identified, thus confirming the reliability of the technique. Figure 1 displays a panel of Northern blots corresponding to six regulated cDNA clones detected in Val5 cells, together with waf-1 and mdm-2. The clones appear heterogeneous in the level of expression, corresponding to medium to low abundance mRNAs. Inserts corresponding to purified plaques were sequenced at both ends and compared with nucleic acid and protein databases using the NCBI blast server. Three clones showed significant homology to known genes (not shown). Three failed to match any sequence in the databases and to date are to be considered potential new genes. We hereby report the characterization of the insert from clone B99, while the other isolated clones will be described elsewhere.

p53-dependent regulation of B99 mRNA in murine fibroblasts

We analysed the mRNA expression of clone B99 by Northern blot, comparing its regulation in Val5 cells with its expression in the recipient Balb/c (10)1 and in Balb/c Val135(25–26) cells. The latter is a cell line derived from p53-deficient Balb/c (10)1 fibroblasts, stably expressing the temperature sensitive (ts) p53 mutant (Val135) additionally carrying a double point mutation in the transactivation domain that renders it transcriptionally inactive (Lin *et al*., 1994). As reported in Figure 2A, column 2, B99 mRNA was strongly induced in Val5 cells after 12 h at the permissive temperature, with a decrease after 24 h. No significant variations in the mRNA levels of B99 were observed in the recipient p53-null cells at 32 or 37°C

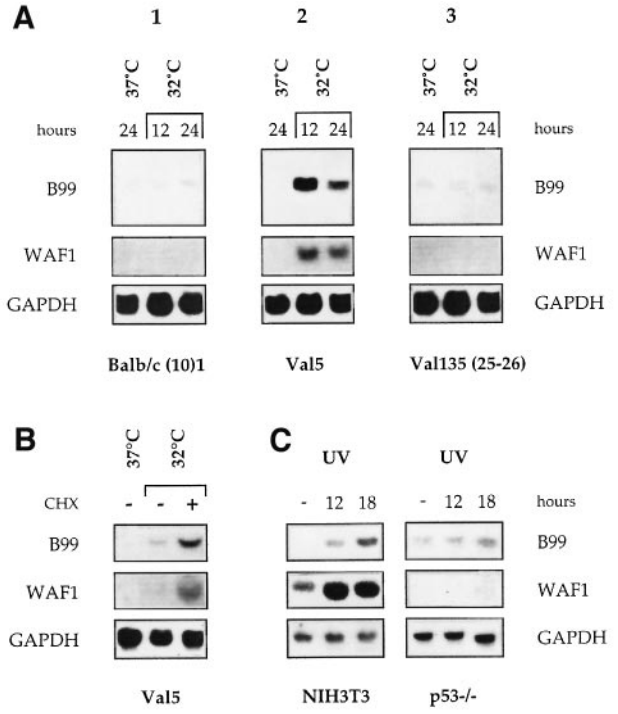

Fig. 2. Northern blot analysis of B99 mRNA expression in murine cell lines. 10 μg of total RNA were loaded on each lane and hybridization was performed under high-stringency conditions. Blots were hybridized with a GAPDH probe as a loading control. (**A**) Regulation in Val5 and control cell lines. RNA was prepared from the indicated cell lines cultured at 37°C or maintained at 32°C for the indicated time. Balb/c (10)1 are murine fibroblasts with both p53 alleles deleted. Val5 are Balb/c (10)1 cells stably expressing the ts Val135 mutant of murine p53. Val135(25–26) are Balb/c (10)1 cells stably transfected with a derivative of the ts p53 Val135 mutant carrying two additional point mutations (residues 25 and 26) that abrogate its transcriptional activation function. (**B**) Regulation in the absence of protein neosynthesis. Total RNA was prepared from Val5 cells grown at 37°C or kept for 6 h at 32°C in the absence or in the presence of 5 μg/ml of the protein synthesis inhibitor cycloheximide. (**C**) Regulation of B99 mRNA in UV-treated mouse fibroblasts. Total RNA was prepared from NIH 3T3 cells or p53–/– mouse embryo fibroblasts at the indicated time points after UV irradiation (10 J/m^2). waf-1 mRNA was analysed in the same experiment as a control of p53 activation. Levels of B99 and waf-1 are not comparable since exposure times were different.

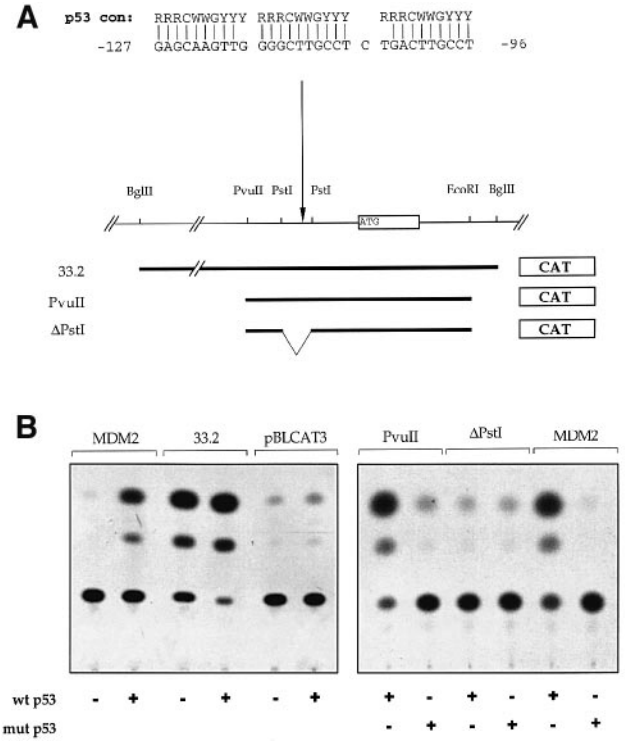

Fig. 3. Identification of a p53-responsive element within the B99 gene. (**A**) Schematic representation of the genomic clone analysed and structure of the CAT reporter constructs. The position of the p53-responsive site identified is indicated, together with its alignment to the consensus p53-binding sequence (El-Deiry *et al*., 1992). (**B**) p53 responsiveness of B99 promoter fragments. The constructs indicated were cotransfected in p53-null Balb/c(10)1 cells with either wild-type p53, mutant p53 or empty expression plasmids. CAT activity was assayed 24 h after transfection. An MDM-2 promoter construct (pBP100CAT) was used as a positive control.

(Figure 2A, column 1), thus indicating that upregulation is not a consequence of temperature shift. Importantly, no induction of B99 mRNA was observed in Val135 (25–26) cells shifted to 32°C (Figure 2A, column 3), where p53 is in wt conformation, binds to target sites on DNA, but is incapable of transcriptional activity (Lin *et al*., 1994), thus suggesting that transactivation by wt p53 is essential for enhanced expression of this clone. As a control, the same Northern blots were hybridized with a waf-1 probe; induction of waf-1 in Val5 was observed both after 12 and 24 h at 32°C, while no waf-1 mRNA could be detected at either 37 or 32°C in both the Balb/c (10)1 or the Val135(25–26) cells.

To test whether induction of B99 might be a secondary consequence of p53 activation, expression of B99 mRNA was analysed in Val5 cells in the absence of *de novo* protein synthesis. No significant B99 mRNA stabilization could be detected upon cycloheximide treatment of Val5 cells maintained at 37°C (not shown). As can be observed in Figure 2B, B99 mRNA was clearly induced after 6 h at 32°C both in the absence or in the presence of 5 μg/ml cycloheximide, as was waf-1 mRNA, analysed as a control. Under these conditions protein synthesis was efficiently inhibited, as determined by the lack of p21Waf1 protein induction observed by immunofluorescence (not shown). We conclude that *de novo* protein synthesis is not required for induction of B99 transcription thus providing further evidence that B99 might be a transcriptional target of p53 in these cells.

To test whether B99 might be regulated by p53 under more physiological conditions, B99 mRNA expression was analysed in mouse fibroblasts subjected to stimuli known to activate endogenous p53 function. For this purpose NIH 3T3 cells, which are wild-type for p53 (Hermeking and Eick, 1994; Del Sal *et al*., 1995), and fibroblasts from p53-nullizygous (p53–/–) mice were treated with UV light (10 J/m^2) and B99 mRNA levels were analysed by Northern blot at 12 and 18 h post-irradiation. As shown in Figure 2C, significant induction of B99 mRNA expression was detected in UV-irradiated NIH 3T3 cells as compared with the basal level in untreated cells. On the contrary, B99 mRNA was not significantly induced by UV treatment in p53–/– MEFs. As a control, waf-1 mRNA was analysed in the same experiments as a marker of p53-dependent transcriptional activity. Thus, UV-induced DNA damage causes upregulation of B99 mRNA in NIH 3T3, but not in p53-null fibroblasts.

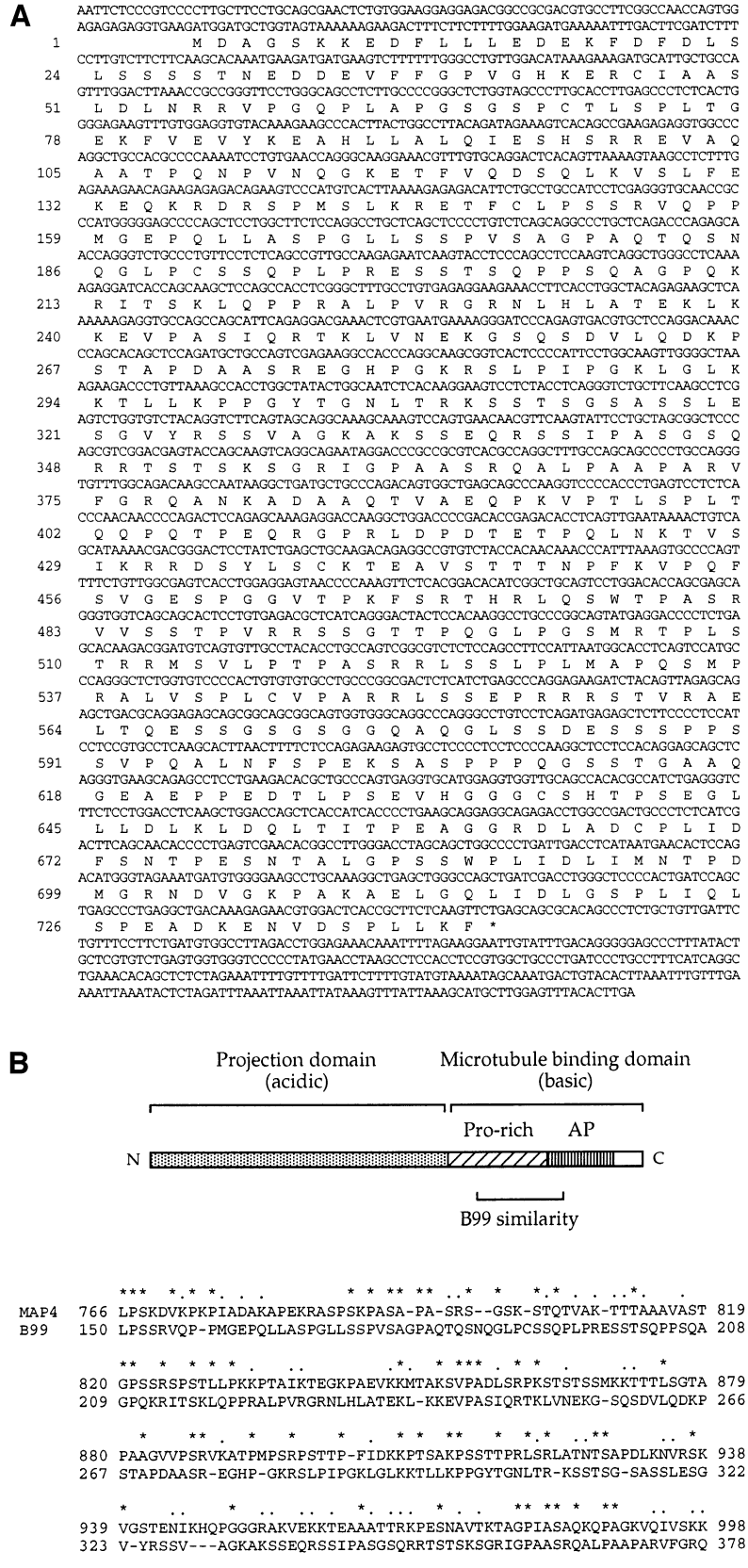

Fig. 4. (**A**) cDNA sequence of clone B99. The amino acid sequence was obtained by conceptual translation of the cDNA, starting at nucleotide 96 and terminating at nucleotide 2319. (**B**) Schematic structure of MAP4 and similarity with B99. MAP4 is divided into an N-terminal projection domain and a C-terminal region involved in interaction with microtubules, further subdivided in a conserved proline-rich domain, and an Assembly Promoting domain (AP) containing the sequences required for MAP4-microtubule interaction (Ookata *et al.*, 1995 and references therein). The alignment between B99 and human MAP4 in the region of similarity is also reported.

Identification of a p53-responsive element within the B99 gene

To better characterize the p53 dependency of B99 transcriptional regulation a screening was performed on a mouse genomic library. A clone was selected containing the 5′ end of the cDNA. Restriction digests of this clone were Southern blotted and hybridized at low stringency with a labelled degenerated oligonucleotide probe corresponding to the consensus p53 binding sequence described by El-Deiry (1992). A 2.8 kb *Bgl*II fragment resulted positive to hybridization; this fragment was isolated and fully sequenced. This genomic segment contained the most 5′ sequence of B99 cDNA and its structure is schematically summarized in Figure 3A. An intron was found starting at nucleotide 26 after the first ATG and extending to the 3′ end of the analysed fragment. A tripartite sequence conforming to the consensus p53 binding site was identified at position –127 from the ATG. Interestingly this sequence contains three half-site decamers, separated respectively by 0 and 1 nucleotides (Figure 3A).

The entire *Bgl*II fragment and corresponding 5′ deletions were cloned in front of a CAT reporter. The constructs were transfected in p53-null Balb/c (10)1 cells alone or together with an expression vector containing either wt p53 or the KH215 mutant version (Finlay *et al*., 1988). As shown in Figure 3B, the genomic segment (33.2) contains a powerful promoter, capable of driving abundant expression of the reporter gene in (10)1 cells. As expected, cotransfection of wt p53 enhanced such expression. Further deletions abolished constitutive transcription from this promoter, unveiling a strong regulation by p53. As shown in Figure 3B, a shorter segment (*Pvu*II) starting at nucleotide –312 with respect to the first ATG was sufficient to confer p53-dependent expression to the CAT reporter. This transactivation was strictly dependent on wt p53 function and was not observed with a mutant p53 protein. When the p53 binding sequence was removed by restriction (Δ*Pst*I) the observed regulation was lost (Figure 3B). We can conclude that a functional p53-responsive element is located in the close vicinity of the B99 promoter, providing the molecular basis for the observed regulation by p53.

B99 encodes a novel protein whose expression is regulated by wt p53

Several signals corresponding to clone B99 were isolated during the screening. Of 30 plaques analysed, two appeared to contain a near full length cDNA, predicted to be ~2.8 kb by Northern blot analysis. DNA sequencing revealed an open reading frame encoding a polypeptide of 741 amino acids, with an ATG codon at position 96 and an in-frame termination codon at nucleotide 2319 (Figure 4A). The protein is rich in positively charged residues and has a predicted isoelectric point of 10.17. Homology search of protein databases revealed no strong similarities to any known gene product. A low homology was found with the proline-rich domain of microtubule-associated protein MAP4 (Figure 4B), a region which has been reported to mediate MAP4 interaction with cyclin B (Ookata *et al*., 1995).

An affinity-purified rabbit polyclonal antibody was obtained against the central part of B99 (amino acids 255–474) produced as a 6×His-tagged bacterially-expressed recombinant protein. In order to characterize biochemically the expression of B99 protein in Val5 cells, a kinetic analysis was performed by Western blot on extracts prepared 12 and 24 h after temperature shift at 32°C. Extracts were also prepared 6, 12 and 24 h after shifting the temperature back to 37°C (i.e. p53 to a mutant conformation), a condition in which the cells exit p53-induced arrest and promptly re-enter the cell cycle. As reported in Figure 5A, the antibody detected a specific protein with an apparent molecular weight of 110 kDa. B99 protein was strongly induced after 12 h at 32°C, while at the 24 h time point a noticeable decrease could be observed, in line with the regulation of B99 mRNA (Figure 2). A polyclonal antibody to p21Waf1 was used in the same analysis as a control of p53 activation.

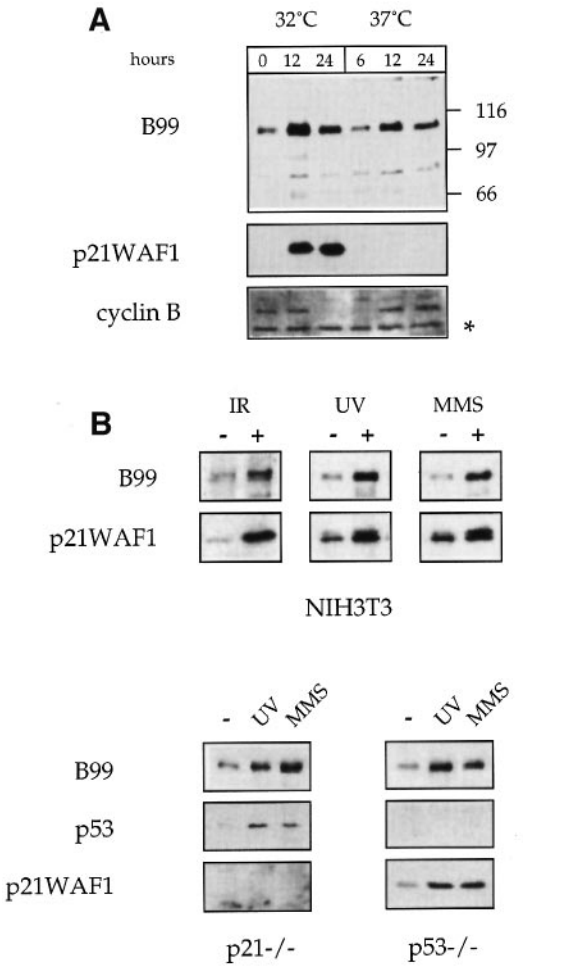

Fig. 5. Western blot analysis of B99 protein expression in murine cells. (**A**) B99 protein regulation in Val5. Cells grown at 37°C for 18 h after plating (time 0) were shifted to 32°C for 12 and 24 h. Cells arrested by 24 h culture at 32°C were then shifted back to 37°C for the indicated times. Cyclin B was analysed as a marker of cell-cycle. The asterisk indicates a cross-reacting protein that serves as internal loading control. (**B**) Accumulation of B99 protein upon DNA damage. NIH 3T3 fibroblasts were treated with 400 rad ionizing radiation (IR), MMS (100 μg/ml for 4 h), or UV-light (10 J/m^2). Primary fibroblasts (MEF) from p53 and p21 knockout mice were exposed to UV-light or MMS. Cells were collected 18 h (UV and MMS) or 24 h (IR) after treatment, and total lysates were analysed by Western blotting with the indicated antibodies.

When Val5 cells were shifted back to 37°C, B99 protein expression was efficiently downregulated within 6 h, slightly accumulating again at 12 h and returning to basal levels after 24 h. For a preliminary understanding of the transient re-appearance of B99 after release from p53-mediated cell-cycle arrest, cyclin B was analysed in the same blot as a marker of G_2 phase. As shown in Figure 5A, cyclin B was efficiently downregulated at 32°C, as expected in arrested cells. When Val5 cells were returned to 37°C, cyclin B re-appeared at 12 h, as observed for B99. This observation suggests that a transient increase in B99 might be associated with passage through the G_2 phase during cell-cycle re-entry.

B99 protein expression was also analysed upon DNA damage in mouse fibroblasts with or without functional p53. Cells were exposed to UV light, ionizing radiation or the alkylating agent methyl methane sulfonate (MMS). Total lysates were prepared 18 h after treatment and B99 protein levels were analysed by immunoblotting. As reported in Figure 5B, B99 clearly accumulated upon DNA damage in NIH 3T3 and p21–/– MEF, which are wt for p53. Interestingly, B99 protein also accumulated in MEF from p53–/– mice, suggesting that B99 might be subject to multiple regulations. Since B99 mRNA appears not to be increased by UV treatment in the same p53–/– fibroblasts (Figure 2C), it is likely that the observed B99 accumulation is due to translational or post-translational regulation. Significantly, as shown in Figure 5, the same behaviour was also observed for p21Waf1. These results indicate that similarly to Waf-1, B99 can be specifically induced by wt p53 but can also respond to other signalling pathways.

B99 protein is localized to the microtubule network

The affinity purified anti-B99 polyclonal antibody was employed to determine the intracellular localization of B99 protein by indirect immunofluorescence. In Val5 cells cultured at the permissive temperature for 12 h, the antibody to B99 revealed a distribution coincident with the microtubule network as defined by anti-tubulin staining (Figure 6a and b), suggesting that B99 protein may be associated with the microtubules. This localization was reproducibly observed both when the cells were fixed with cold methanol or 3% paraformaldehyde. Ectopic expression of B99 in Balb/c (10)1 mouse fibroblasts confirmed the specificity of the signal; as shown in Figure 6c and d, the transfected exogenous B99 protein colocalized with tubulin, displaying a distribution similar to the endogenous protein observed in Val5 cells at 32°C.

Endogenous B99 is expressed in the G_2/M fraction of the cell population

When Val5 cells were shifted to the permissive temperature of 32°C and B99 induction was observed by immunofluorescence, expression appeared heterogeneous in the cell population analysed. Figure 7A shows two fields of Val5 cells cultured at 37°C or kept for 12 h at 32°C and stained with the affinity-purified anti B99 antibody; as can be seen, B99 protein was clearly induced, but not in all the cells. Double immunofluorescence staining revealed that there was no correlation between B99 expression and fluctuations in the levels of p53 within individual cells (not shown). A flow-cytometric analysis was therefore performed to measure the DNA content of the B99-expressing sub-population of Val5 cells as shown in Figure 7B. Fixed cells were stained with the anti-B99 antibody followed by a FITC-conjugated anti-rabbit antibody, and DNA was stained with propidium iodide (PI). An appropriate gating was applied to the specific protein fluorescence, based on the background signal detected in the 37°C sample. In the 32°C sample, the gated B99 positive cells displayed a 4N DNA content, indicating that B99 expression was restricted to the G_2/M subpopulation. The same correlation could also be observed at longer periods after temperature shift (i.e. 24 and 48 h), when Val5 still display a significant, albeit lower, fraction of cells with 4N DNA content (not shown). It should be stressed here that the outgated B99-negative cells showed a markedly lower 4N fraction with respect to the total population (see Figure 7B), indicating that the majority of G_2 cells expressed high levels of B99 protein.

To understand whether the observed specificity of induction could be extended to a system in which p53 activation was more physiological, expression of endogenous B99 protein was analysed in NIH 3T3 mouse fibroblasts upon γ-radiation treatment. As previously described, irradiation triggers a significant induction of B99 (Figure 5B). We used a complementary approach to observe the possible correlation between G_2 phase and B99 induction already noticed in Val5 cells; the FACS was employed to separate G_1 and G_2/M cells to >95% purity, as described in Delia *et al*. (1997). NIH 3T3 cells were exposed to 400 rad and grown for an additional 24 h, after which Hoechst 33342 dye was added to the culture medium. The cells were then separated on the basis of their DNA content, and expression of B99 protein was analysed by Western blot on lysates of sorted cells. Figure 8B shows the DNA content distribution of the total irradiated cell population and the corresponding sorted fractions. Equal amounts of total proteins were loaded on each lane. As can be observed in Figure 8A, basal levels of B99 protein were detected in the G_2/M fraction of untreated NIH 3T3 cells. Most notably, upon γ-irradiation of the same cells the observed induction of B99 protein was restricted to the population with a 4N DNA content. Such selectivity of expression appears to be specific for B99 protein, since p21Waf1 was induced to similar levels in all the fractions (Figure 8A).

Ectopically expressed B99 delays G_2/M phase progression

To gain insight into the possible function of B99, an expression vector carrying the B99 cDNA was transfected in murine Balb/c (10)1 and NIH 3T3 cells. Western blot analysis on transfected (10)1 cells confirmed the expression of the exogenous protein, with an electrophoretic mobility similar to the endogenous B99 protein from Val5 (not shown). When the B99 expression plasmid was cotransfected with a vector expressing the neomycin resistance gene and cells were selected in the presence of G418, no stably expressing clones could be obtained in both cell lines, indicating that B99 might have a growth-suppressive effect. Transiently transfected cells were therefore analysed by indirect immunofluorescence together with Hoechst DNA staining. Observation of the nuclear morphology of B99-positive cells failed to reveal con-

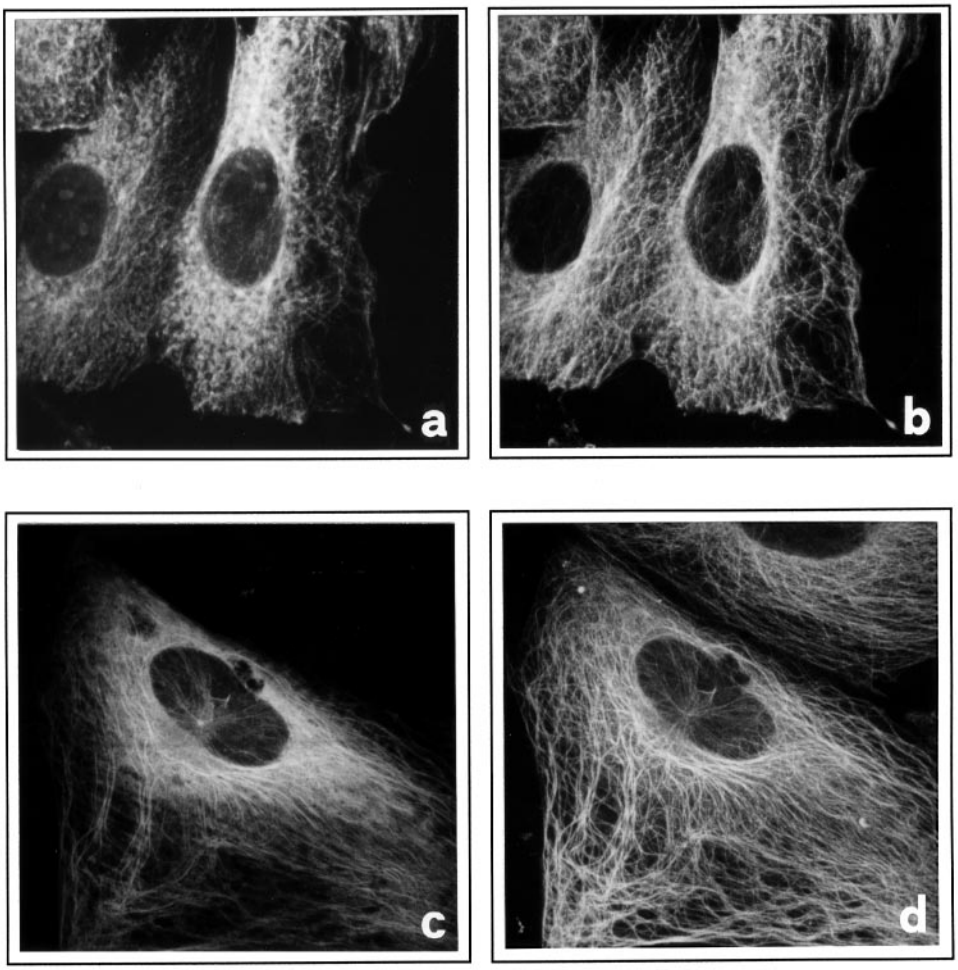

Fig. 6. Immunological definition of B99 endocellular localization. B99 protein was detected using a rabbit anti-B99 affinity-purified polyclonal antibody followed by a FITC-conjugated anti-rabbit antibody. Tubulin was stained using a monoclonal antibody (Sigma) followed by RITC-conjugated anti-mouse antibody. Images were taken with a laser scan confocal microscope. (**a**) Detection of endogenous B99 protein in Val5 cells cultured at 32°C for 12 h. (**b**) The same microscopic field as in (a), stained for tubulin. (**c**) Detection of ectopically expressed B99 protein in p53-null Balb/c (10)1 cells 48 h after transfection. (**d**) The same microscopic field as in (c), stained for tubulin.

densed chromatin and collapsed nuclei indicative of apoptosis (not shown). A transient transfection assay was finally employed to determine the cell-cycle distribution of B99-overexpressing cells by flow cytometry. Cells were collected 48 h after transfection, ethanol-fixed, and stained for B99. DNA was stained PI and the DNA content of B99-expressing cells was determined by application of the appropriate gating on a biparametric cytofluorimetric analysis (Figure 9A). The profile of a representative experiment in Balb/c (10)1 mouse fibroblasts is shown in Figure 9B, where box D shows the gated, B99-overexpressing sub-population in relation to the total cell population reported in box C. As can be observed, the cells staining positive for B99 display a larger fraction with a 4N DNA content, indicative of a prolonged G_2/M phase. As a control the same cells were transfected with a Gas2 expression vector (Brancolini *et al*., 1995) and as shown in box F, overexpression of the microfilament associated Gas2 protein had no influence on the cell-cycle profile of these cells. The same results could also be obtained in NIH 3T3 and human SAOS-2 osteosarcoma cells (not shown). To rule out the possibility that the observed cell-cycle distribution of transfected cells might be a consequence of specific instability of the B99 protein during G_1, we confirmed that comparable levels of expression could be obtained in Balb/c (10)1 cells that were arrested at the G_1/S boundary by aphidicoline treatment (not shown). Altogether, these data suggest that B99 overexpression delays the G_2 phase of the cell cycle.

Discussion

Intense research is ongoing to detect and characterize genes that are regulated by wt p53 with the view that essential functions of this fundamental tumour suppressor are mediated by transcriptional activation of effector genes. In the present study we report the isolation of novel transcripts that are upregulated by wt p53 in a murine cellular system carrying an inducible p53 allele. These cells undergo an efficient p53-dependent arrest with no evidence of apoptosis at the permissive temperature, and this arrest is readily reversible upon inactivation of p53 by returning to the non-permissive temperature (Del Sal *et al*., 1996; Murphy *et al*., 1996). This experimental system should therefore select for genes involved in the cell-cycle regulatory function of wt p53, and specifically

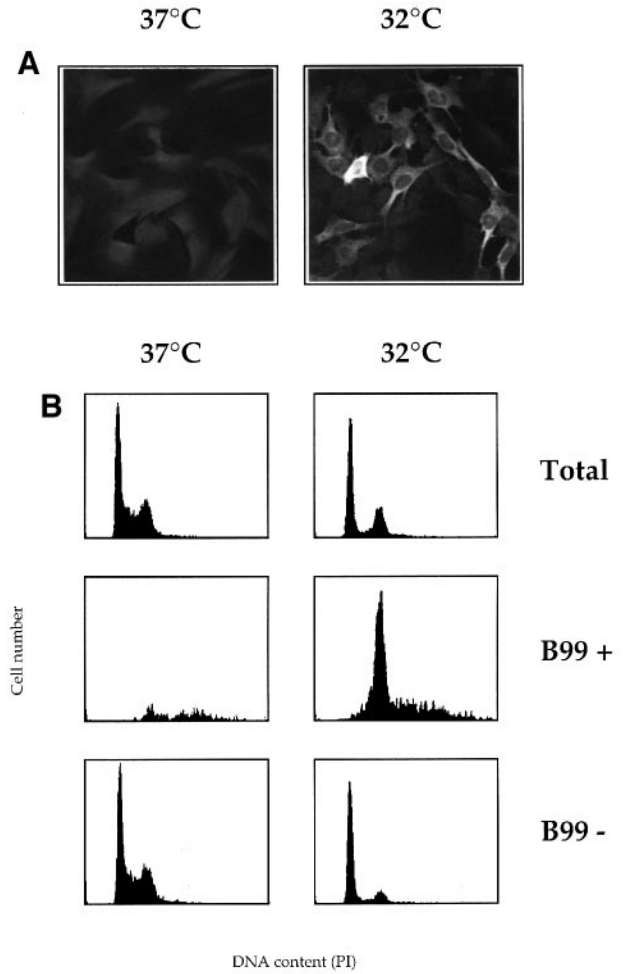

Fig. 7. B99 is selectively expressed in the G_2/M fraction of arrested Val5 cells. (**A**) Expression of endogenous B99 protein in Val5 fibroblasts. Val5 cells growing at 37°C or kept for 12 h at 32°C were stained with the affinity-purified anti B99 antibody followed by a FITC-conjugated anti-rabbit secondary antibody. (**B**) Flow-cytometric analysis of Val5 cells corresponding to the same growth conditions as in (A). A gating on the specific protein fluorescence was applied to sort the cells expressing high levels of B99 (B99+) from the total population. The profile of the outgated, B99 negative cells is also reported (B99–).

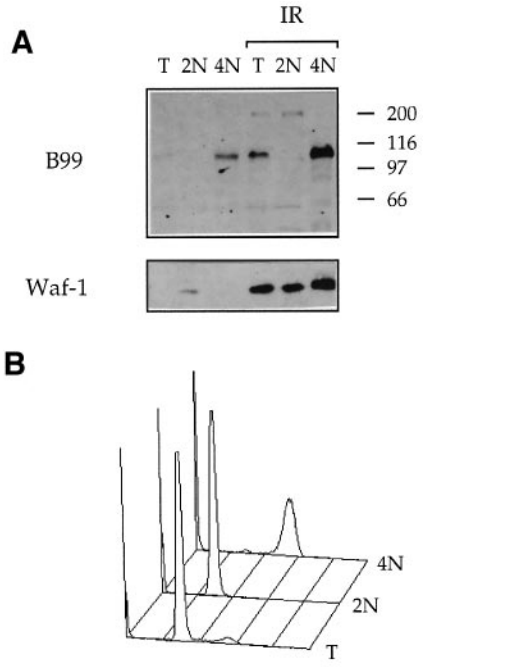

Fig. 8. B99 is selectively induced in the G_2/M fraction of gamma-irradiated NIH 3T3 cells. (**A**) Western blot analysis of total (T), G_0/G_1 (2N), or G_2/M (4N) cells separated by FACSvantage sorting on the basis of DNA content before or 24 h after IR treatment (400 rad). Detection of B99 and p21Waf1 proteins was performed as described in the legend to Figure 4. (**B**) DNA content profile of the cell fractions before (T) and after sorting (2N and 4N). The diagram indicates the efficiency of separation. Only the sample corresponding to irradiated cells (IR) is shown.

in maintaining a reversible growth arrest. A differential hybridization screening with subtracted cDNA probes provided us with six regulated transcripts.

Here we have described a cDNA encoding a novel protein, B99, that is efficiently upregulated in a wt p53-dependent manner in at least two different systems: B99 mRNA and protein are upregulated by DNA damage in murine fibroblasts, and are strongly induced in Val5 cells by activation of wt p53 in the absence of DNA damage. We identified a consensus p53-binding site within the sequences of the B99 gene, and demonstrated that this element is sufficient to confer wt p53-dependent expression to a reporter gene. Taken together, these data indicate that B99 is a direct transcriptional target of wt p53.

Interestingly, despite the fact that B99 mRNA was not upregulated upon UV treatment in p53-null cells, we observed induction of B99 protein by DNA damage in p53–/– fibroblasts, indicating that B99 is subject to both p53-dependent and -independent regulation. A similar behaviour was also observed for the p53-inducible gene p21Waf1, which appears to be regulated during cell cycle and upon DNA damage even in the absence of functional p53 (Michieli *et al*., 1994; Macleod *et al*., 1995; Loignon *et al*., 1997).

When B99 was induced by activation of latent p53 in Val5 cells, it was selectively expressed in the subpopulation with a 4N DNA content. We could also demonstrate that endogenous B99 was specifically induced in the G_2/M fraction of NIH 3T3 fibroblasts exposed to DNA damage. It is worth noting that B99 was not accumulated in Balb/c (10)1 or NIH 3T3 cells arrested at G_2/M by microtubule-active drugs such as nocodazole or taxol (L.Collavin, unpublished data), thus indicating that B99 upregulation is not a consequence of G_2/M arrest, but actually requires specific signals. To our knowledge, this is the first report of a p53-inducible gene with G_2-specific expression. This selectivity of induction suggests that B99 might be transcriptionally regulated by p53 in a cell-cycle-dependent manner. Several authors reported that binding of p53 to responsive elements in the promoters of different target genes can be modulated by phosphorylation of specific residues on the protein (Hecker *et al*., 1996 and references therein). It has also been shown that p53 can be phosphorylated by different CDKs, and that such phosphorylation modulates the DNA binding activity of p53 and the selectivity of target site recognition, thus suggesting that the transcriptional function of p53 might be different during different phases of the cell cycle (Wang and Prives, 1995). The hypothesis that B99 promoter might

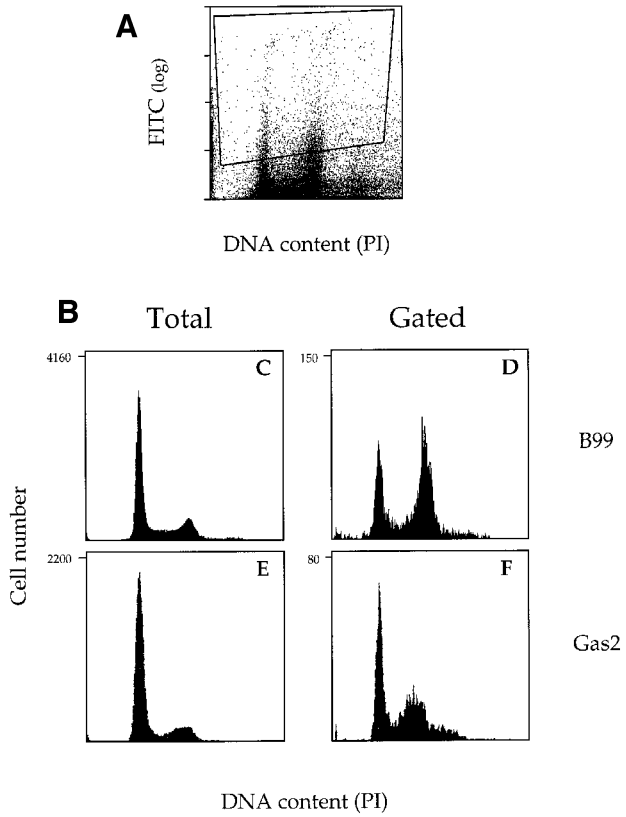

Fig. 9. Cell-cycle analysis of cells transiently transfected with B99. Balb/c (10)1 fibroblasts were transfected with expression vectors containing B99 or Gas2 under control of the SV40 promoter. Cells were collected 48 h after transfection, fixed in cold ethanol, incubated with appropriate antibodies and with PI (to stain cellular DNA) and analysed by flow cytometry. (**A**) Example of a representative analysis on B99-transfected cells. B99-specific staining (FITC) was plotted as a function of DNA content (PI). An appropriate gating was applied to differentiate the transfected population from the bulk of untransfected cells as shown by the marked region in the figure. (**B**) Cell-cycle profile of transfected cells. (**C**) and (**E**) profile of the total cell populations, obtained by applying no gate. (**D**) and (**F**) DNA content distribution of the gated subpopulations of B99 or Gas2-overexpressing cells.

be regulated by p53 in a cell-cycle-dependent fashion is an interesting issue that will be subject of future work.

Finally, when B99 was transfected in p53-null murine fibroblasts it showed a growth-suppressive effect and interfered with G_2/M phase progression, as documented by the increased fraction with 4N DNA content in cells overexpressing exogenous B99.

Few hints on the possible biochemical function of B99 protein could be drawn from its primary sequence and intracellular localization. B99 protein is 741 amino acids long and is rather basic. It was shown to localize to the microtubule network but lacks obvious microtubule interaction motifs. Further biochemical characterization will be required to determine if B99 interacts directly with tubulin and has to be considered a proper microtubule-associated protein (MAP). B99 protein fails to reveal strong homologies to any peptide in the databases, but shares a region of weak similarity with MAP4. Such similarity resides within the C-terminal half of MAP4 (Figure 4), and more specifically in a domain which is not directly involved in binding to tubulin. Rather, this region was shown to mediate MAP4 interaction with cyclin B (Ookata *et al*., 1995). Interestingly, wt p53 has been reported to repress specifically MAP4 transcription in Val5 fibroblasts, and p53-mediated downregulation of MAP4 has been functionally correlated with p53-dependent apoptosis (Murphy *et al*., 1996). Accordingly, MAP4 overexpression was shown to interfere with p53-induced apoptosis, but was reported to have no effect on cell-cycle arrest (Murphy *et al*., 1996). Thus, the microtubule-localized B99 protein is induced by wt p53 under the same conditions in which the microtubule-associated MAP4 is downregulated, suggesting the intriguing possibility of a functional interplay between these two proteins.

Recently, accumulating evidence indicates an involvement of p53 in the control of cell-cycle events that occur past the G_1/S transition. Indeed, a complex pattern of still poorly characterized wt p53-dependent functions is being gradually revealed through various experimental approaches. Loss of p53 function has been clearly correlated with abnormal centrosome amplification (Brown *et al*., 1994; Fukasawa *et al*., 1996). It was shown that p53 can activate a G_2/M cell-cycle block (Agarwal *et al*., 1995; Stewart *et al*., 1995). And it has been clearly demonstrated that wt p53 is required for proper function of a checkpoint preventing S-phase entry in cells that fail to traverse mitosis in the presence of spindle-inhibitory drugs (Cross *et al*., 1995; Lanni and Jacks, 1998). It has been reported that such p53-dependent spindle checkpoint requires p21Waf1 induction (Lanni and Jacks, 1998), but previous work suggested that it was still functional in fibroblasts from p21 nullizygous mice (Deng *et al*., 1995). It has been proposed that p53-induced G_2 arrest could be mediated through accumulation of p21Waf1, which binds the mitotic cdc2/B kinase and could be inhibiting MPF activity (Xiong *et al*., 1993; Li *et al*., 1994; Agarwal *et al*., 1995;). Moreover, it has also been proposed that G_2/M cell-cycle arrest could result from p53-dependent induction of 14-3-3σ protein, a negative regulator of cdc25c (Furnari *et al*., 1997; Hermeking *et al*., 1997).

Nevertheless, despite many recent advances, the emerging picture is still far from being complete, and it is reasonable to think that other gene products might be involved in p53-dependent regulation of G_2/M-specific events.

In the inducible p53 system used in the present work, when Val5 cells are arrested by culture at 32°C a significant number of cells still display a 4N DNA content (Figure 7). As previously shown for other cellular systems, it is likely that p53 is arresting or delaying these cells in the G_2/M phase of the cell cycle. Interestingly, B99 is selectively induced by p53 in those cells. Furthermore, when B99 was ectopically expressed in proliferating cells, it interfered with G_2/M progression. On the basis of the above observations we propose B99 as a candidate mediator of G_2-specific functions of wt p53.

Materials and methods

Cell lines and culture conditions

All the cell lines were routinely cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml). Gamma irradiation was performed using a ^{137}Caesium source as described in Delia *et al*. (1997). UV treatment consisted of a 10 J/m^2 irradiation as described in Del Sal *et al*. (1996). MMS (Sigma) was added to the

culture medium at a final concentration of 25–100 μg/ml for 4 h, after which the medium was replaced and cells grown for an additional 18 h before harvesting.

Differential screening with subtracted probes

Total RNA was extracted from adherent cells according to Chomczynsky and Sacchi (1987). Poly $(A)^+$ RNA was isolated using the Oligotex-dT kit (Quiagen) and treated with DNaseI before purification and precipitation. cDNA libraries from Val5 cultures growing at 32°C (12 and 24 h) were constructed employing a cDNA Library Construction Kit (Clontech). Double-stranded cDNAs were cloned in the λ-ZAP Express Vector (Stratagene) and packaged using Gigapack II Gold extracts (Stratagene). To synthesize the cDNA probes, 1 μg of poly (A)+ RNA from Val5 cultured at 32°C (target) or 37°C (driver) was used as template for cDNA synthesis in the presence of 75 μM random hexamer primers and 0.5 mCi of [α-^{32}P]dCTP. cDNAs were purified using the Prep-A-Gene Matrix (Bio-Rad), treated with proteinase K, precipitated and resuspended in hybridization solution. Each cDNA probe was then mixed with 0.66 μg/μl Poly$(A)^+$ and 0.33 μg/μl total RNA from the opposite growth condition, and hybridized in a total volume of 15 μl for 36 h at 68°C in a sealed glass capillary. After hybridization the single-stranded fractions were separated though a hydroxylapatite column at 60°C. The single-stranded fractions were used directly as probes. During the screening 250 000 plaques of the 32°C specific cDNA library were plated and two lifts were made from each dish on nylon membranes (Colony/Plaque Screen, Dupont). The first lift from each plate was hybridized with the driver cDNA probe, and the second was hybridized with the target cDNA probe. Hybridization and washes were performed at high stringency conditions. Clones were excised *in vivo* according to the manufacturer's protocol (Stratagene).

For Northern blotting, RNA samples were separated on 1% agarose gels containing 2.6% formaldehyde. RNA was transferred to nylon membranes according to the protocol described in Chomczynski (1992).

DNA sequencing

DNA sequencing was performed on an automated fluorescent DNA sequencer (European Molecular Biology Laboratory; EMBL) using the T7-Sequencing Kit (Pharmacia). Sequence assembly and analysis was done using the Geneskipper V1.1 software (EMBL).

Genomic screening and CAT assays

Approximately 500 000 plaques of a mouse genomic library (Stratagene) were screened with a 5′ end fragment from B99 cDNA. Restriction digests of positive clones were Southern blotted and hybridized at 40°C in 6× SSC/1% SDS with a ^{32}P-labelled degenerated oligonucleotide probe corresponding to the following sequence: 5′-(A/G) (A/G) (A/G)C(A/T) (A/T)G(C/T) (C/T) (C/T) (A/G) (A/G) (A/G)C(A/T) (A/T)G(C/T) (C/T) (C/T)-3′. Washes were performed at 37°C in 0.2× SSC/0.1% SDS.

For reporter assays, genomic fragments were cloned in the pBLCAT3 vector (Luckow and Schutz, 1987). Balb/c (10)1 cells plated at low density in 3 cm Petri dishes were transfected by the calcium-phosphate precipitation technique with 3 μg of promoter constructs plus 0.6 μg of p53 expression plasmids (pMSVcL-wt p53 and pMSVKH215-mutant p53) and empty vector to reach a total of 6 μg. Cells were collected 24 h later and analysed for CAT activity by routine procedures (Sambrook *et al*., 1989).

Production of B99-specific antiserum

A fragment from clone B99 corresponding to amino acids 255–474 of the B99 protein was cloned in-frame in the polylinker of the pQE-9 vector (Qiagen). The protein was expressed and purified according to the QIAexpressionist System kit (Qiagen). Rabbits were injected with 200 μg of recombinant protein mixed 1:1 with complete Freund's adjuvant (Sigma). The same amount of protein was subsequently injected every 21 days for 2 months. The B99 antibody was purified by affinity chomatography on a B99-coupled AffiPrepA matrix (Bio-Rad).

Immunoblotting

Cellular lysates were transferred to 0.2 μm pore size nitrocellulose membrane (Schleicher & Schuell) using a semidry blotting apparatus (Bio-Rad). Detection of B99 protein was performed in high salt conditions (5% dry-milk, 50 mM Tris pH 7.5, 1 M NaCl, 0.1% Tween-20) for 2 h at room temperature (RT). All other antigens were detected in standard conditions. p21Waf1 was detected with a polyclonal rabbit serum raised against a Waf-1 synthetic peptide (Del Sal *et al*., 1996). Murine p53 was detected using the PAb240 monoclonal antibody (Oncogene Research). Primary antibodies were detected by incubation for 1 h with horseradish peroxidase (HRPO)-conjugated secondary antibodies (Southern Biotechnology). Blots were developed with the ECL chemoluminescence system (Amersham).

Immunofluorescence microscopy

Cells were plated on glass coverslips within 3 cm tissue culture dishes. After washing with PBS, cells were fixed for 15 min in cold methanol (–20°C). Alternatively, cells were fixed in PBS/3% PFA for 20 min at RT, treated with PBS/1% glycine for 5 min, and permeabilized in PBS/0.1% Triton-X 100 for 5 min. B99 protein was stained using the rabbit affinity-purified anti-B99 antibody followed by goat anti-rabbit FITC-conjugated antibody (Sigma). Tubulin was stained using a monoclonal anti-β-tubulin antibody (Sigma) followed by goat anti-mouse RITC-conjugated antibody (Southern Biotechnology). Nuclei were stained with Hoechst 33342. After incubations and extensive washes, coverslips were mounted on glass slides and analysed using a laser scan confocal microscope (Zeiss).

Cell sorting

Live G_0/G_1 and G_2/M fractions (purity >95%) were obtained as described in Delia *et al*. (1997). Briefly, cells were stained by addition of Hoechst 33342 (10 μg/ml) to the culture medium and subsequently sorted on a FACSvantage instrument (Becton-Dickinson) equipped with a 5 W argon-ion laser emitting in the UV spectrum (352 nm), with a cooling system for both the sample and collection holders.

Transfection and flow-cytometric analysis

The B99 cDNA was subcloned in the pGDSV7 mammalian expression vector, under control of the SV40 promoter (Del Sal *et al*., 1995). Cells were transfected with 7.5 μg of pGDSV7-B99 or pGDSV7-Gas2 plasmids by the calcium phosphate precipitation procedure (Sambrook *et al*., 1989). Approximately 10^5 cells were plated on 6 cm Petri dishes and 8 h later the DNA precipitate was added. After 12 h the precipitate was replaced with DMEM containing 10% FCS, and the cells cultured for additional 48 h. After washing with PBS, cells were harvested by trypsin treatment and fixed with 70% ethanol at –20°C. Fixed cells were washed in PBS/5% FCS and incubated with the polyclonal anti-B99 affinity-purified antibody or affinity-purified anti-Gas2 antibody (Brancolini *et al.*, 1995) for 30 min at 37°C. Immune complexes were detected by incubation for 30 min with a goat FITC-conjugated anti-rabbit antibody (Sigma) and DNA was stained with 25 μg/ml PI after RNaseA treatment. Flow cytometric analyses were performed either on a Bryte HS (Bio-Rad) or a FACSvantage (Becton Dickinson) cytofluorimeter.

Accession number

The cDNA sequence of clone B99 has been submitted to the DDBJ/EMBL/GenBank databases under accession No. AJ222580.

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