Cell-cycle regulation of the p53-inducible gene B99

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Abstract B99 is a p53-inducible gene whose accumulation upon p53 activation is restricted to late S/G2 cells. Here we have analyzed B99 regulation during the cell cycle in murine cells with or without functional p53. We report that B99 accumulates in late S/G2 phase, is phosphorylated in mitosis, and disappears in G1 phase, regardless of the status of p53. As a complement to this observation, we show that B99 is not induced by p53 in quiescent cells. Therefore, B99 expression is modulated both by cell-cycle regulatory mechanisms and by p53, and p53 can increase the cellular levels of B99 only during the window of the cell cycle when it is normally expressed. On the basis of these observations we rename B99 Gtse-1 (G-two- and S-phase-expressed).

Key words: B99; p53-inducible gene; Cell cycle; G2 phase-specific; Gtse-1

1. Introduction

The tumor suppressor gene p53 is one of the most frequent targets of genetic alteration in human cancer [1]. It encodes a short-lived transcription factor which exerts a strong antiproliferative activity in response to a wide spectrum of different stimuli such as DNA damage, oncogene activation or depletion of ribonucleotides [2,3]. p53 suppresses cell growth by inducing cell-cycle arrest or apoptosis [3,4] and this activity plays a crucial role in preventing genetic instability and cellular transformation [5].

It is well established that p53 can efficiently block the cell cycle at the G1/S transition, this effect being largely dependent on induction of p21Waf1, a potent inhibitor of G1 cyclin-dependent kinases [6,7]. But increasing evidence suggests that p53 might also have an important function in controlling cell-cycle events past the G1/S transition. In fact, p53-null cells tend to develop an aberrant number of centrosomes [8,9] and have a defective spindle checkpoint [10], and in several cell lines p53 activation results in both G1 and G2 arrest [11–13]. The importance of p21Waf1 in these activities is unclear [14,15] and it is likely that some other p53 target genes might be involved [16,17].

B99 was discovered during a differential screening devised to isolate novel p53-inducible genes in a murine cell line (Val5) expressing the temperature-sensitive Val135 mutant p53 [18]. Upon temperature-mediated p53 activation, Val5 cells undergo a reversible cell-cycle arrest without evidence of apoptosis [19,20]; it is therefore reasonable to assume that genes induced by p53 in these cells are likely to be involved in establishing and maintaining a status of reversible growth arrest. Preliminary characterization revealed that B99 encodes a novel protein localized to the microtubules, with no significant homology to any gene in the databases. The B99 promoter contains a functional p53-responsive element and B99 was shown to be induced by p53 under a number of different conditions [18]. Interestingly, flow cytometric analysis revealed that p53 can induce accumulation of B99 protein only in cells bearing a 4N DNA content, and transfection experiments suggested that B99 overexpression delays progression through G2 phase [18]. Taken together, these data point to B99 as an appealing candidate effector for G2-specific functions of wild-type (wt) p53.

To our knowledge, B99 is the first example of a p53 target gene induced by p53 only within a specific cell-cycle window. This G2 specificity of p53-dependent induction prompted us to analyze in more detail the regulation of B99 during the cell cycle, in the presence and in the absence of functional p53.

2. Materials and methods

2.1. Cell culture conditions and Northern blotting

Cells were routinely cultured at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml). The cell lines NIH3T3 (wt p53), BALB/c(10)1 (p53-null), and Val5 (temperature-sensitive p53 Val135) were previously described [25]. For Northern blotting, approximately 10 μg of total RNA was separated on 1% agarose gels containing 2.6% formaldehyde and transferred to nylon membranes as described in [18]. UV treatment consisted of 10 J/m^2 irradiation as described in [18]. Mitotic cells were prepared after 16 h incubation in the presence of 0.125 μg/ml nocodazole. Culture medium was discarded to eliminate floating dead cells. Metaphase cells were detached by vigorous shaking in phosphate-buffered saline (PBS) supplemented with 0.125 μg/ml nocodazole and collected by centrifugation. Microtubule-active drugs were purchased from Sigma and used at the following concentrations: nocodazole 0.125 μg/ml; colcemid 0.5 μg/ml; paclitaxel (taxol) 1 μM.

2.2. Flow cytometry

Subconfluent cells were harvested by trypsin treatment and fixed with 70% ethanol at −20°C. Fixed cells were washed in PBS/5% FCS and incubated for 30 min at room temperature either with the anti-B99 antiserum [18], or with a pre-immune rabbit serum as a control. Immune complexes were detected by incubation for 30 min with a FITC-conjugated goat anti-rabbit antibody (Sigma) and DNA was stained with 25 μg/ml propidium iodide after RNase A treatment. Samples were analyzed on a Bryte HS flow cytometer (Bio-Rad).

2.3. Plasmids, transfections and luciferase assays

The previously described 33.2 genomic fragment containing the B99

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promoter [18] was cloned in the promoterless pGL2-basic vector (Promega) to generate the p33.2-LUC construct. Subconfluent cells were transfected with the pGL2-basic, the pGL2-promoter (Promega), or the p33.2-LUC vectors, together with a 1/8 amount of pBABE-Puro, using Lipofectin (Life Technologies). Transfected cells were selected for about 2 weeks in 5 μg/ml puromycin. Surviving clones from each plate were trypsinized and pooled together. Luciferase assays were performed using the Promega Luciferase Assay Kit according to the manufacturer's instructions. Luciferase activity was measured in a Turner Design luminometer (Promega) and light values were normalized for the protein concentration in each sample as determined by the Bradford colorimetric assay (Bio-Rad).

2.4. Immunoblotting and immunoprecipitation

For immunoblotting, cells were scraped in Laemmli buffer, sonicated, and immediately boiled. Lysates were fractionated by SDS–PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell) using a semi-dry blotting apparatus (Bio-Rad). Detection of B99 protein was performed in high salt conditions (5% low-fat dry milk, 50 mM Tris–HCl pH 7.5, 1 M NaCl, 0.1% Tween-20) for 2 h at room temperature. Other antigens were detected in standard conditions. Primary antibodies were detected by incubation for 1 h with horseradish peroxidase-conjugated secondary antibodies (Southern Biotechnology). Blots were developed with the ECL chemiluminescence system (Amersham). For immunoprecipitation, cells were lysed in buffer A (50 mM Tris–HCl pH 8, 200 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM dithiothreitol, 100 mM NaF, 1 mM sodium orthovanadate, 50 μg/ml phenylmethylsulfonyl fluoride, and 10 μg/ml chymostatin, leupeptin, antipain and pepstatin), passed several times through a 27 gauge needle, and centrifuged to remove cell debris. Supernatants were preclarified with protein A-Sepharose for 30 min, and incubated with MPM-2 monoclonal antibody (mitotic phosphoprotein monoclonal-2, kindly provided by T. Stukenberg) prebound to 20 μl of protein A-Sepharose CL-4B (Amersham Pharmacia) for 3 h at 4°C with gentle agitation. Beads were washed four times with buffer A, and bound proteins were eluted by boiling in Laemmli sample buffer. Immunoprecipitated proteins and aliquots of the supernatants were separated on 10% SDS–PAGE and analyzed by immunoblotting.

2.5. In vitro translation and incubation in Xenopus egg extracts

B99 was in vitro translated from the pCS2B99 expression vector [21] using a coupled transcription/translation reticulocyte lysate system (Promega) in the presence of ^{35}S-labelled methionine. 1 μl of in

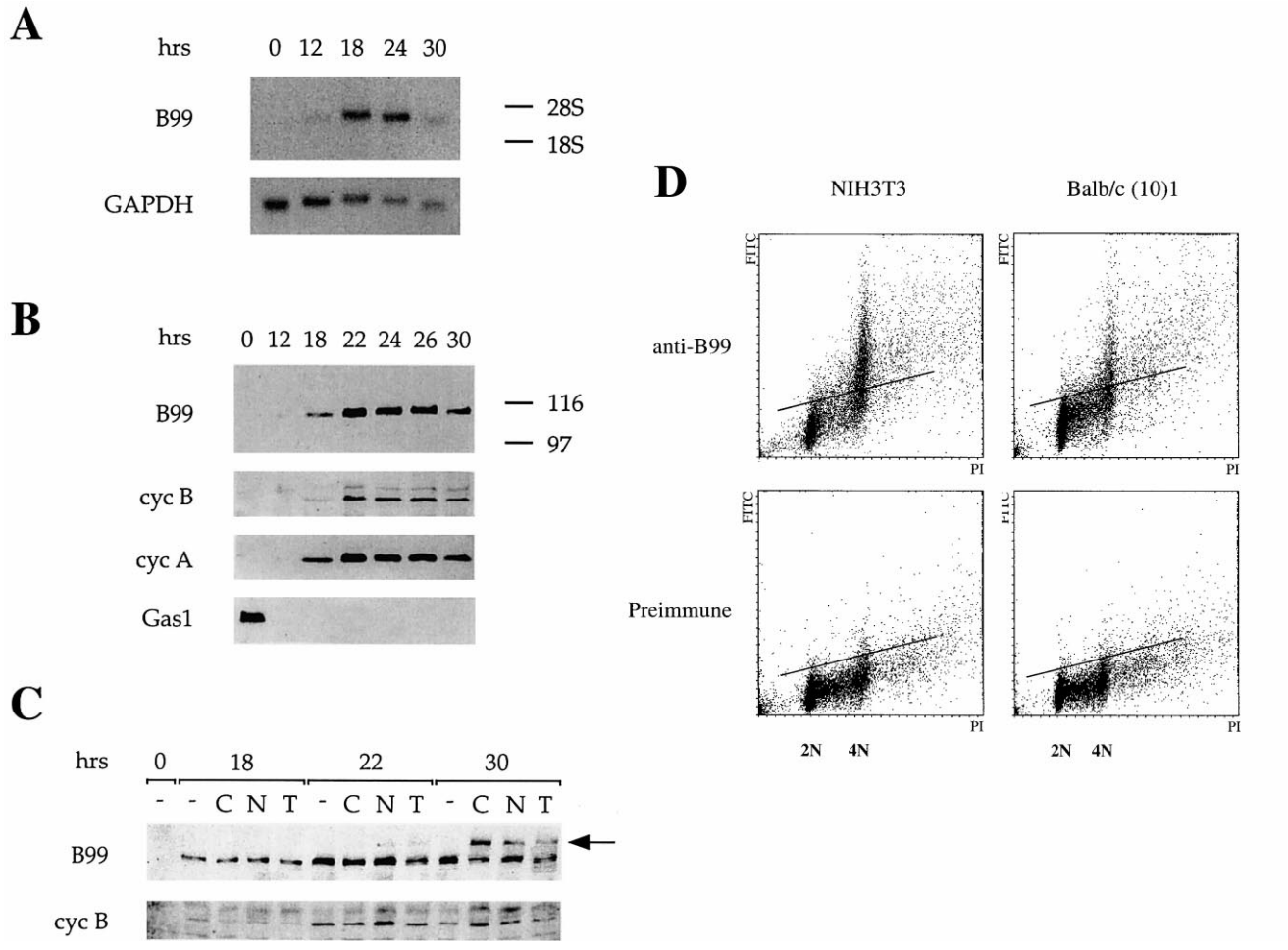

Fig. 1. Analysis of B99 expression during the cell cycle. A: Cell-cycle regulation of B99 mRNA. NIH3T3 fibroblasts were arrested by 48 h culture in low serum (0.5% FCS) and stimulated to re-enter the cell cycle by addition of 20% FCS. Total RNA was prepared at the indicated time points and B99 expression was analyzed by Northern blotting. GAPDH was analyzed in the same blot as a loading control. B: Cell-cycle regulation of B99 protein. NIH3T3 cells were treated as in A. Lysates were collected at the indicated time points and B99 expression was analyzed by immunoblotting. Cyclins A and B were analyzed in the same lysates as markers of cell-cycle progression. The growth arrest-specific protein Gas1 was analyzed as a marker of G0 [23]. C: Cell-cycle regulation of B99 is not affected by microtubule-active drugs. NIH3T3 cells were treated as in A in the absence or in the presence of microtubule-active drugs. Lysates were prepared at the indicated time points and B99 was detected by immunoblotting. Cyclin B was analyzed as a marker of G2/M. C: colcemid; N: nocodazole; T: taxol (paclitaxel). The arrow indicates a slower-migrating form of B99 specifically observed in mitotic cells. D: Bivariate flow cytometric analysis of B99 protein and DNA content. Subconfluent NIH3T3 and BALB/c(10)1 cells were simultaneously stained with propidium iodide (*x*-axis) and either the anti-B99 or a control antibody followed by a FITC-conjugated secondary antibody (*y*-axis). FITC fluorescence is plotted against DNA content. Values are expressed on a linear scale. The line indicates the approximate cut-off for B99-positive cells.

vitro translated B99 was added to 10 μl interphase or mitotic extract (at room temperature) and time points were taken at 0 min and 60 min. Calf intestinal phosphatase was added at 60 min, and samples were incubated for an additional 30 min at room temperature. Interphase extracts were prepared as described [22], using calcium ionophore at 1 μg/ml to activate unfertilized *Xenopus* eggs to complete the second meiotic division. Cycloheximide was added at a final concentration of 100 μg/ml to inhibit further translation of B99 and to prevent accumulation of cyclins (i.e. to maintain the interphase state during the incubation period). Mitotic extracts were prepared by addition of 60 μg/ml bacterially expressed sea urchin δ-90 cyclin B (a non-degradable form of cyclin) to the interphase extracts [21].

3. Results

3.1. Cell-cycle regulation of B99 mRNA and protein

B99 mRNA and protein levels were analyzed in NIH3T3 mouse fibroblasts arrested in G0 by serum starvation and stimulated to synchronously re-enter the cell cycle by addition of 20% FCS [23]. As shown in Fig. 1A,B, B99 was undetectable in quiescent cells, accumulated during S phase (18 h), and reached maximum levels during G2 (22 h), with kinetics very similar to G2 cyclins. The protein expression profile correlated with expression of the mRNA. Notably, both B99 mRNA and protein levels clearly decreased at 30 h when, although synchronization is significantly loosened (see expression of cyclins), a large fraction of the cells are in the G1 phase of the following cell cycle.

Since B99 protein consistently displays microtubular localization [18], we asked whether its expression could be affected by the status of microtubules. B99 protein was analyzed in NIH3T3 cells arrested in G0 by serum starvation and stimulated to synchronously re-enter the cell cycle in the presence of microtubule-active drugs. As reported in Fig. 1C, B99 protein was regulated normally in the presence of nocodazole or colcemid, with a somewhat lower induction in the presence of taxol, probably because of the higher toxicity of the drug. Expression of B99 paralleled expression of cyclin B analyzed in the same extracts. Therefore, expression of B99 is not significantly affected by the status of the microtubules. A lower mobility band appeared in lysates highly enriched in mitotic cells (30 h time point); this band reflects a post-translational modification of B99 which is described below.

As a complement to the data obtained during cell-cycle reentry from quiescence, we employed flow cytometry to analyze B99 levels during the normal cell cycle in asynchronous proliferating cells. Subconfluent NIH3T3 and BALB/c(10)1 fibroblasts were stained with the anti-B99 antiserum and B99 fluorescence was plotted against DNA fluorescence as shown in Fig. 1D. As expected, B99 fluorescence was low in the G1 phase and peaked in the G2 phase, confirming the regulation observed during cell-cycle progression from G0. A similar profile was reported for cyclin B1 in human lymphoblastoid cells [24]. This regulation was virtually identical in NIH3T3, which are wild-type for p53 [25], and in BALB/c(10)1, which are p53-null [26], indicating that such regulation is not dependent on p53.

3.2. Cell-cycle regulation of the B99 promoter

The analysis of B99 mRNA (Fig. 1A) suggested that B99 might be transcriptionally regulated during the cell cycle. To address this issue, a 2.6-kb genomic fragment encompassing the promoter sequences and the first exon of B99 was cloned upstream of firefly luciferase in the pGL2-basic vector (p33.2-LUC). This construct was co-transfected with a puromycin resistance gene in NIH3T3 and in BALB/c(10)1 fibroblasts. After selection, resistant clones were pooled and luciferase activity was measured in proliferating and quiescent cells. As a control, pools of stable transfectants were selected containing either a promoterless (pGL2-basic) or an SV40-driven (pGL2-promoter) luciferase reporter vector. As summarized in Fig. 2A, the B99 promoter was significantly repressed in cells arrested either by serum starvation or by growth to confluence. In contrast, expression from the SV40 promoter underwent only minor variations. Similar results were obtained in transient transfection experiments in both cell lines (not shown). Again, although this genomic construct contained a functional p53-responsive element [18], the behavior of the B99 promoter was identical in p53-containing and in p53-null cells, indicating that the observed regulation was not a consequence of cell-cycle fluctuations of p53 activity.

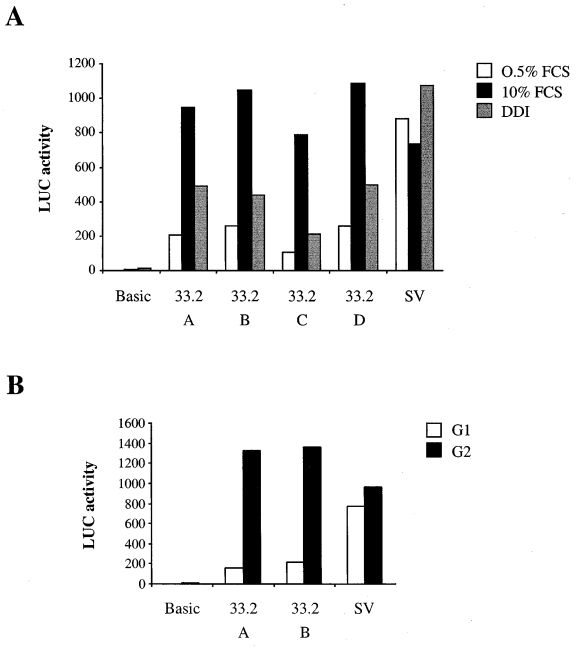

Fig. 2. Cell-cycle regulation of B99 promoter. A: Polyclonal stable transfectants containing the B99 promoter construct p33.2-LUC were selected in NIH3T3 and BALB/c(10)1 murine fibroblasts (33.2 A to D: two separate polyclones were obtained in each cell line). Stable transfectants containing either the promoterless pGL2-basic vector (Basic) or the SV40-driven pGL2-promoter vector (SV) were selected as negative and positive controls respectively. Luciferase activity was measured in subconfluent growing cells (10% FCS), and in cells arrested by 48 h serum starvation (0.5% FCS) or growth to confluence (DDI). Clones 33.2 A and B, as well as Basic and SV controls, are NIH3T3 cells. Clones 33.2 C and D are BALB/c(10)1 cells. B: The indicated polyclones were growth-arrested by serum starvation and stimulated to synchronously re-enter the cell cycle as described in the legend to Fig. 1. Luciferase activity was measured 6 h (G1) and 22 h (G2) after serum addition.

Transcription from B99 promoter was also analyzed during synchronous cell-cycle re-entry of serum-starved NIH3T3 (Fig. 2B). Activity of the B99 promoter was low in G1 (6 h after serum addition) and high in G2 (22 h), thus recapitulating the observed regulation of B99 mRNA and protein.

3.3. B99 is modified by phosphorylation in mitotic cells

To analyze the regulation of B99 protein during the transition from M to G1 phase, mitotic NIH3T3 cells were accu-

mulated by nocodazole treatment, collected by shake-off, and re-plated in the absence of nocodazole [27]. As shown in Fig. 3A, the antibody to B99 detected in mitotic cells a protein displaying significantly slower mobility than interphase extracts. This mitosis-specific mobility shift of B99 is due to post-translational modification, since it was also detected on transiently transfected epitope-tagged B99 (not shown). And this modification is not a consequence of drug treatment, since it was also observed when mitotic cells were collected by shake-off without prior nocodazole treatment (not shown). When metaphase cells are re-plated in the absence of nocodazole, they rapidly complete mitosis and enter the G1 phase. As shown in Fig. 3A, B99 protein disappeared within 2 h after re-plating, confirming that B99 is efficiently downregulated in G1.

To understand the molecular basis of the observed mobility shift in mitotic cells, in vitro translated B99 protein was incubated in interphase or mitotic *Xenopus* egg extracts. As shown in Fig. 3B, B99 protein was shifted to lower mobility in the mitotic extract. This band migrated with approximately the same electrophoretic mobility as the endogenous B99 protein detected in mitotic cells (see Fig. 3A). The protein returned to its original mobility after treatment with phosphatase, implying that the shift is due to phosphorylation. Similar results were obtained when total lysates from mitotic NIH3T3 cells were phosphatase-treated and B99 protein was analyzed by immunoblotting (not shown).

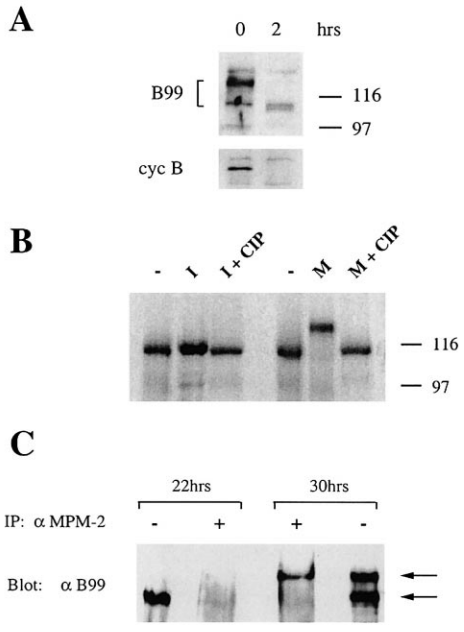

Fig. 3. B99 phosphorylation in mitotic cells. A: Analysis of B99 during M to G1 transition. Mitotic NIH3T3 cells were collected by shake-off after nocodazole treatment (time 0) and replated in drug-free medium for 2 h. B99 protein was analyzed by immunoblotting together with cyclin B as a marker of G2/M. Note the altered migration of B99 in the mitotic lysate. B: Phosphorylation of B99 in mitotic extracts. Radioactively labeled in vitro translated B99 was incubated for 1 h in interphase (I) or mitotic (M) *Xenopus* egg extracts at room temperature. Where indicated, calf intestinal phosphatase (CIP) was added after 1 h incubation, and incubated for an additional 30 min. C: Immunoprecipitation of mitotic B99 with the MPM-2 antibody. NIH3T3 cells were arrested by serum starvation and stimulated to enter the cell cycle as described in Fig. 1. Transition through mitosis was inhibited by nocodazole and cells were collected 22 h or 30 h after serum stimulation. Proteins were immunoprecipitated (IP) with the MPM-2 antibody in the presence of phosphatase inhibitors, followed by immunoblotting with the anti-B99 serum. Interphase and mitotic forms of B99 are indicated by arrows.

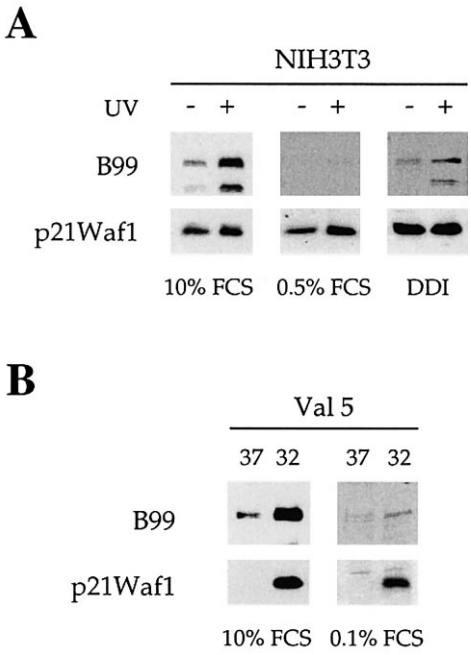

Fig. 4. p53-dependent B99 induction in cycling versus arrested cells. A: Analysis of B99 expression after UV irradiation of NIH3T3 cells asynchronously growing (10% FCS), or arrested by either serum starvation (0.5% FCS) or growth to confluence (DDI). Lysates were prepared 18 h after UV treatment. B: Analysis of p53-dependent regulation of B99 in Val5 cells asynchronously growing (10% FCS) or arrested by serum starvation (0.1% FCS). For each culture condition, lysates were prepared from cells growing at 37°C or kept for 12 h at 32°C. p21Waf1 was analyzed in the same extracts as a control of p53 activation. Bands in different blots are not comparable because of different exposure times required to increase the very weak B99 signal in arrested cells.

B99 protein contains several Ser/Thr-Pro motifs; the antibody MPM-2 recognizes the phosphorylated Ser/Thr-Pro epitope on many important regulators specifically phosphorylated at mitosis [28,29]. We therefore asked whether mitotic B99 could be recognized by the MPM-2 antibody; quiescent NIH3T3 were stimulated to re-enter the cell cycle by addition of growth factors, nocodazole was added after 18 h, and mitotic phosphoproteins were immunoprecipitated with MPM-2 at 30 h post-activation. Under these conditions the interphase and mitotic forms of B99 are both easily detected (see Fig. 1C). As shown in Fig. 3C, the MPM-2 antibody selectively immunoprecipitated the slower-migrating, mitotic form of B99. The interphase form of B99 was not recognized by MPM-2 in these extracts, or in similar extracts from G2 cells in which the mitotic form is absent. We conclude that B99 is specifically phosphorylated during mitosis to generate an MPM-2 phosphoepitope.

3.4. B99 is not induced by p53 in quiescent cells

Since B99 mRNA and protein are dramatically induced by p53 activation and are strictly regulated during the cell cycle, we further investigated the correlation between cell cycle-dependent and p53-dependent regulation of B99. To analyze B99 protein levels after activation of wt p53 in conditions in

which B99 is not normally expressed, we tested B99 induction after DNA damage in quiescent NIH3T3. Dividing and arrested cells were exposed to UV light (10 J/m^2) and lysates were prepared 18 h after treatment. As shown in Fig. 4A, B99 protein was efficiently accumulated in asynchronously growing cells. Induction of B99 was undetectable in serum-starved, and extremely weak in density-inhibited cells. In contrast, regulation of p21Waf1, analyzed as a control, was less influenced by growth conditions. It is important to note that under these conditions UV damage to NIH3T3 cells does not increase the G2/M subpopulation (data not shown). Anyway, it is possible that p53 activity might fluctuate during the cell cycle [30–34], and it has been reported that UV-induced p53 accumulation might not occur in G0 cells, although DNA binding and sequence-specific transactivation are still detected [31,35]. We therefore analyzed B99 expression after activation of a temperature-sensitive p53 protein (Val135) constitutively expressed at high levels in the Val5 cell line [19,36]. Val5 fibroblasts were made quiescent by prolonged culture in 0.1% FCS, a treatment resulting in a significant decrease of S/G2 cells as assessed by flow cytometry (not shown). p53 was subsequently activated by transferring the cells to the permissive temperature of 32°C and B99 expression was analyzed by immunoblotting 12 h later. As shown in Fig. 4B, B99 was strongly induced after conformational activation of p53 in proliferating cells, but was very poorly induced in arrested cells. This regulation was specific, since p21Waf1 was clearly induced in both conditions.

4. Discussion

B99 was cloned as a p53-inducible gene, its promoter contains a functional p53-responsive element, and B99 protein is efficiently induced upon p53 activation by a number of diverse stimuli, but only in cells having a 4N DNA content [18]. This last observation prompted us to investigate whether B99 might be normally regulated during the cell cycle and how such a regulation might be interlaced with its p53 responsiveness. We have now clearly demonstrated that B99 is tightly regulated during the cell cycle, its expression beginning at S phase and reaching maximum levels in G2. Importantly, we have observed the same regulation for the recently cloned human homologue of B99 (manuscript in preparation). During mitosis B99 is phosphorylated, and promptly disappears as cells complete anaphase and enter G1. This regulation is identical in wt p53-containing and p53-null murine cells. We have shown that a genomic fragment containing the B99 promoter is sufficient to confer cell-cycle regulation to a luciferase reporter gene and again, we provided evidence that this regulation is uncoupled from p53 function. Detailed molecular analysis of the promoter elements mediating cell cycle-specific transcription of B99 was beyond the aim of the present study, but the construct we have described is a useful tool for future analysis. In particular, it will be interesting to determine whether any transcriptional regulatory elements are conserved between B99 promoter and those of other cell cycle-regulated genes such as CDC25C, Plk1, cyclin A and cyclin B1, which share a similar profile of expression [37–39].

Analysis of B99 in mitotic cells revealed that B99 is modified during mitosis, resulting in a large shift in electrophoretic mobility. Although we cannot exclude additional modifications, we presented data clearly indicating that B99 protein is phosphorylated in mitotic extracts. In a number of proteins, many of which are associated with structural components of the mitotic apparatus, phosphorylation generates a phosphoepitope which is specifically recognized by the MPM-2 monoclonal antibody [28,40]. We have shown here that mitotic B99 is recognized by MPM-2, thus adding B99 to the list of mitotic MPM-2 phosphoepitopes. It is attractive to speculate that such phosphorylation might regulate B99 function during mitosis (e.g. modulating its affinity for microtubules or other protein partners), or might be targeting B99 for subsequent destruction. These hypotheses await experimental verification and will be the subject of future investigations.

During preparation of this article a paper was published describing a novel APC recognition signal functionally similar but distinct from the destruction box of mitotic cyclins [21]; this motif, named the KEN box, behaves as a targeting signal for degradation by the Cdh1-activated form of the anaphase-promoting complex (Cdh1-APC). Interestingly, the authors recognized a consensus KEN sequence within the carboxy-terminus of B99 and demonstrated that in vitro translated B99 is efficiently degraded by Cdh1-APC in *Xenopus* egg extracts [21]. Deletion or mutation of the KEN box protected B99 from ubiquitination and degradation [21]. Since Cdh1-APC assembles at late mitosis and is believed to be active throughout the G1 phase (see [41,42] and references therein), this observation provides a convincing explanation for the rapid disappearance of B99 protein in post-mitotic cells (see Fig. 3A).

Many p53-inducible genes are subject to p53-independent regulation; although these genes are induced by p53, they have autonomous cellular roles in the absence of p53 activation. A clear example is p21Waf1, a very important and well studied effector of p53 functions [7,43] which is normally expressed in p53-null cells, plays an autonomous and p53-independent role in cell-cycle progression, and is regulated by a number of different pathways in addition to p53 [44–46]. Here we show that the same principle holds true for B99; in fact not only is B99 normally regulated during the cell cycle in p53-null fibroblasts, but B99 protein was detected under proliferating conditions in all murine cell lines tested so far, regardless of their p53 status (L. Collavin and M. Monte, unpublished). Expressed sequence tags corresponding to B99 have been sequenced from two-cell mouse embryo (accession number AU018143) and mouse blastocyst (accession number AA571318) libraries, further suggesting that B99 is expressed in most, if not all, dividing cells. Finally, similarly to p21Waf1, B99 protein can be induced by UV treatment in p53-null fibroblasts [18].

We therefore conclude that B99 is a cell cycle-regulated gene which is only expressed in S and G2 phase. Within this window of expression, B99 levels can be dramatically increased by activation of p53. We think this supports the hypothesis that B99 might be involved in cell cycle-related functions, or checkpoints, during late S and G2 phases. On the basis of these observations, we propose replacing the provisional name B99 with the definitive name Gtse-1, acronym for G-two- and S-phase-expressed protein.

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