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Transcriptional dependent activities of wild type p53: characterisation of two novel p53-inducible genes

Thesis submitted for the Degree of Doctor Philosophiae

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Foreword

This Thesis is centered on the characterisation of two novel p53-inducible genes, which were previously cloned in our group through a differential subtractive-hybridisation approach. These two genes encode unrelated proteins; I have been studying them as independent projects, and I therefore discuss them as separate pieces of work in PART 2 and PART 3.

Since this Thesis essentially treats p53-dependent transactivation, the introductory chapter (PART 1) is principally focused on p53 transcriptional-dependent activities. In particular, I tried to systematically review the current knowledge about cellular genes induced by wt p53. More comprehensive information about other important aspects of p53 function, which I necessarily just briefly summarised, can be found in a number of excellent reviews and references cited there [80, 90, 93, 119, 120, 133].

During my graduate studies I have also been involved in other projects, eventually resulting in published work. Those publications are included in this Thesis as Xeroxed reprints in PART 4.

Most of the work described in PARTS 2 and 3 is contained in the following publications:

Utrera*, R., Collavin*, L., Lazarevic*, D., Delia, D. and Schneider, C. (1998) A novel p53-inducible gene coding for a microtubule-localized protein with G2-phase-specific expression. *Embo J*, **17**, 5015-25. (* equal contributors)

Collavin, L., Lazarevic, D., Utrera, R., Marzinotto, S., Monte, M. and Schneider, C. (1998) wt p53 dependent expression of a membrane-associated isoform of Adenylate Kinase. *Submitted*

List of Abbreviations

AT	Ataxia-Telangiectasia
DSB	Double Strand Breaks
EMSA	Electrophoretic Mobility Shift Assay
FACS	Fluorescence Activated Cell Sorter
IR	Ionizing Radiation
MEF	Murine Embryonic Fibroblasts
MPF	M-phase Promoting Factor
NES	Nuclear Localization Signal
p53-BS	p53-Binding Site
p53-RE	p53-Responsive Element
PI	Propidium Iodide
REF	Rat Embryonic Fibroblasts
ROS	Reactive Oxygen Species
SAGE	Simultaneous Analysis of Gene Expression
SST	Sequence Specific Transactivation
TR	Transferrin Receptor
UTR	UnTranslated Region
VSV	Vesicular Stomatitis Virus

INTRODUCTION

"In other families, as generation gives rise to generation, father passes to child a cleft in the chin, a love of music; mother passes to child an aptitude for dance, a curve of the cheeks. In one family, the legacy was far different. What was being handed down, unknowingly, was cancer. Or, more precisely, the mutant form of a gene whose inheritance virtually guarantees that a malignant tumor will grow. Grandfather carried the mutation. He developed pancreatic cancer at the age of 48; he died two years later. But first he passed on the mutant gene to his five children. One daughter developed breast cancer at 22 and thyroid cancer at 34. A son had a brain tumor at 54. Another daughter was diagnosed with breast cancer at 32; a year later she was dead. And that was only the first generation."

"For as long as biologists have studied cancer, they have nurtured one dream. Their hope has been that, for all of the many different cancers that can eat away at a body-from the glioblastomas that creep through the brain like kudzu through Georgia, to the lung and colon cancers that envelop vital organs like a death shroud-there will turn out to be a single step that determines whether cells turn cancerous.

By targeting drugs at that step, physicians would be able to stop a cell from heading down the path toward cancer just as a switchman stops a train from going down the wrong track. It turns out that a gene called p53-the gene whose useless, mutant form the luckless family passed from parent to child through the generations-may be that switch. If p53 is healthy and doing its job, it keeps the cell on the track of normalcy. But if the p53 gene is absent, damaged or tied up by other molecules, the cell goes down the track toward cancer."

from: "The Cancer Killer", by Sharon Begley, NewsWeek , Dec 23, 1996.

The p53 protein has been center of extensive study ever since it became clear that more than 50% of human cancers contain mutations in this gene. Specific databases catalogue these mutations, and the data collected up to now indicate that p53 is involved in more than 50 different types of tumors, affecting different cells and tissues [95, 105, 106] and the list is growing. As Sharon Begley wrote on NewsWeek, p53 is perhaps the best candidate to fulfil the biologists' long-sought dream of finding a single gene product involved in the critical step determining whether any cell would turn cancerous. Accordingly, an overwhelming amount of data on p53 has accumulated during 16 years of intense research world-wide (at the moment of writing this manuscript a Medline database search for "p53" returned 12353 hits). Nevertheless, although the understanding of p53 biological functions is rapidly progressing, new findings continuously pose new paradoxes, making the picture even more complex.

An historical perspective on p53 research

The p53 cellular protein was first described in 1979 as a peptide present in very small amounts in normal cells, but in much higher amounts in cells transformed by SV40, as well as in a variety of other tumor-derived cells [127, 139]. Antibodies reactive to p53 were found in sera of tumor-bearing mice and cancer patients [35, 203]; and after molecular cloning of murine p53 it was clearly shown that p53 could efficiently cooperate with a ras oncogene to transform cells in culture [111, 187, 193]. These and many other evidences induced researchers to ascribe p53 in the ever-growing list of cellular oncogenes. Nevertheless, in the following years new discoveries induced researchers to drastically change their thinking. In 1989, after cloning and chromosomal mapping of murine and human p53 genes, it became clear that p53 was mutated in many commonly used cell lines, most notably those from which the cDNA was originally cloned [58, 67]. This meant that gene transfer experiments in which p53 displayed oncogenic behaviour had been performed with a mutant version of the gene. This was not really surprising since many proto-oncogenes become transforming after mutation. The real breakthrough came with the discovery that the wild-type (wt) version of the p53 gene was able to strongly inhibit transformation by cellular and viral oncogenes [59, 66]. At the same time, Vogelstein and co-workers discovered that the p53 locus on chromosome 17 was frequently altered in colorectal cancer, indicating that the p53 gene had clear attributes of a classical tumor suppressor [7]. One year later, germline p53 mutations were discovered in Li-Fraumeni cancer-prone families [155]. And finally, in 1992, the seminal observation that homozygous and hemizygous p53 knockout mice are severely cancer-prone definitely indicated p53 as a fundamental tumor suppressor [51]. In parallel, it became clear that several oncogenic DNA viruses express viral proteins which strongly interfere with the sequence specific transactivation (SST) function of wtp53, and that these proteins are required for the virus to be oncogenic [172, 252, 270]. This further confirmed that abolition of p53 function is a key step in the process of cellular transformation.

In the same years, the massive effort of many different groups resulted in the key discoveries about p53 function which set the stage for all subsequent research. It was shown that wt p53 can induce cell-cycle arrest [164], or induce programmed cell death [260]. It was determined that p53 contains a strong transcriptional activating domain [65], and displays sequence specific DNA binding [116]. It was shown that wt p53 can specifically transactivate cellular genes, and that tumor derived mutants lack this function [199]. This burst of discoveries culminated with the nomination of p53 as "Molecule of the Year" by *Science* in December 1993.

Since then, a large amount of information has accumulated on the cellular events acting upstream of p53 (modulating its stability, cellular localisation, phosphorylation status, selectivity of transactivation), and downstream of p53 (integrating diverse cellular pathways to mediate cell-cycle arrest, DNA repair, or apoptosis), revealing an extremely complex picture which is probably still far from being complete.

p53-related proteins

Very recently, novel proteins similar to p53 have been identified in human and rodent cells, thus suggesting that p53 might be member of a gene family (see [188] and references therein). The p53-like proteins isolated up to date are essentially two, named p73 and p51. They present tissue specificity of expression, and each of them exists in at least two different splice variants (p73α and β, and p51A and B). These p53-related proteins share highest homology with the DNA binding domain of p53, and maintain important functions such as transactivation of at least some p53-inducible genes. None of these p53-related proteins seems to heterodimerise with p53, and none of these proteins appears to be activated in response to DNA damage. The p73 protein has been shown to be a potent inducer of apoptosis, and deletion of the p73 gene was found in human neuroblastomas; these evidences suggested that p73 might be a tumor suppressor [112, 113]. Notably, p73-nullizygous mice display severe neurological, immunological and reproductive defects, but fail to develop spontaneous tumors (D. Caput, 9th p53

workshop, 1998). These preliminary observations would suggest a divergence of functions among p53-family members, with p53 being the key player in preserving genome integrity. Nevertheless, it is conceivable that a degree of functional redundancy might exists, which could eventually explain some of the apparent paradoxes about p53 function [30, 50].

Structure and function of p53 domains

The p53 protein is a tetrameric transcription factor which can be structurally and functionally divided in 5 domains (see Figure 1).

The transactivation domain

The first 42 aminoacids at the N-terminus constitute a strong transcription activating domain which interacts with components of the basal transcription machinery and can stimulate gene expression. Aminoacids 13 to 23 are highly conserved in p53 from diverse organisms, and Leu22 and Trp23 mediate direct interaction with TAFII70 and TAFII31 in vitro [144, 234]; accordingly, mutation of these residues strongly impairs the sequence-specific transactivation function of p53 [137]. Recent studies reported that p300/CBP transcription factor, which has histone acetyl-transferase activity, also interacts with p53 transactivation domain, and that p300 and p53 act as transcriptional co-activators [6, 84]. Thus, p53 uses an hydrophobic domain to interact with the basal transcription machinery. Interestingly, cellular (i.e. Mdm-2) and viral (i.e. E1B 55K) proteins known to inhibit SST function also contact these residues [137]. Additionally, the interaction with Mdm-2 plays a crucial role in regulating cellular localisation and degradation of the protein [194]. Therefore, this domain not only plays a mechanistic role in transcriptional activation but has also a regulatory importance on p53 function.

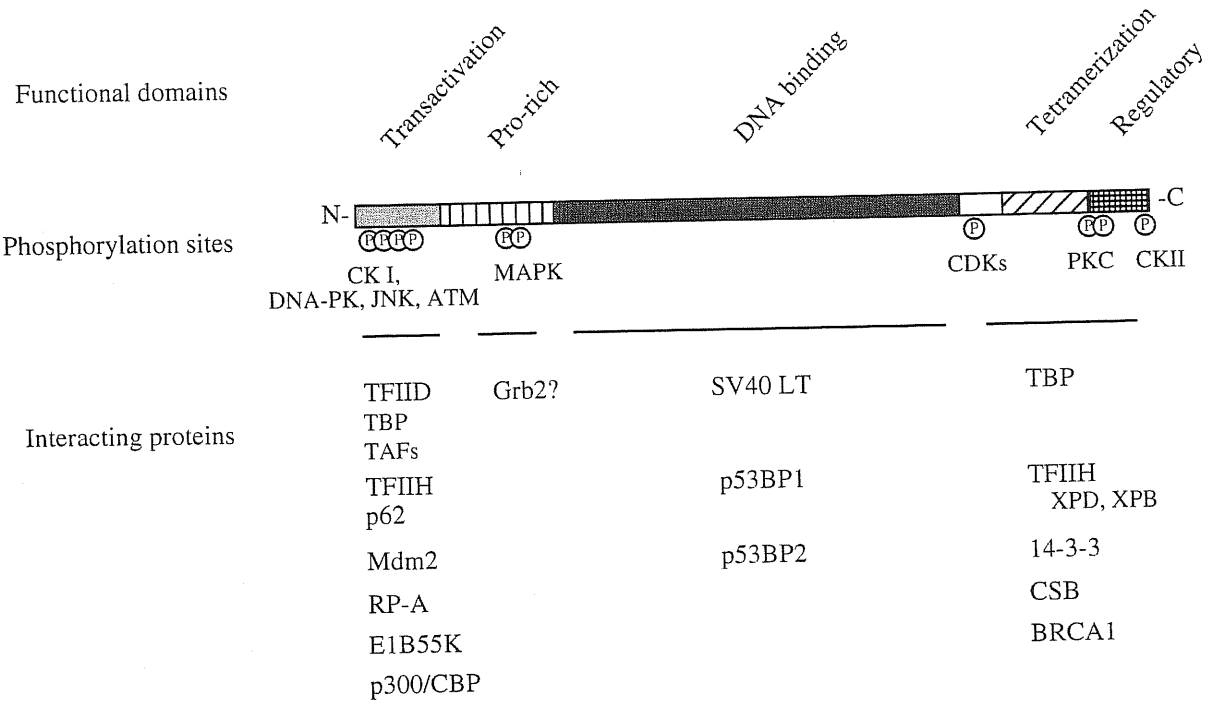


Figure 1
Schematic structure of p53 domains, phosphorylation sites, and known interacting proteins.

(Adapted from Ko and Prives, 1996)

The Proline-rich domain

Aminoacids 42 to 100 constitute a proline-rich domain of undefined three dimensional structure, which contains several repeats of the PXXP motif. Repeats of the PXXP sequence have been reported to be docking sites for SH3 domains in several proteins involved in signal transduction from growth factor receptors [31]. Therefore, it has been proposed that the proline-rich domain in p53 could mediate interaction with some SH3 containing cellular protein(s). Accordingly, a few recent studies suggest that this domain is involved in growth-suppression or induction of apoptosis by p53, likely mediating its transcriptional repression function [206, 208, 244]. It is of great interest the recent observation that the cellular SH3-containing Grb2 protein can interact with p53 at the proline-rich domain (C. Venot, 9th p53 workshop, 1998); Grb2 is involved in growth-factor receptor-activated pathways, and could modulate p53 activity in response to specific signals. A very recent report demonstrated that the poly-proline domain is required for specific transactivation of the pro-apoptotic PIG3 gene promoter [241].

A sequence polymorphism within this domain of p53 is found in the human population, resulting in either a proline or an arginine at position 72. Thus, the p53Arg variant lacks one of the five PXXP motifs present in human p53. Notably, a recent study showed that the p53Arg variant is more susceptible to papillomavirus (HPV) E6-induced degradation than the p53Pro counterpart; strikingly, analysis of a number of cervical tumors revealed a significant correlation between homozygous p53Arg genotype and development of HPV-associated cancers [230].

The DNA-binding domain

Aminoacids 100 to 300 constitute the sequence-specific DNA binding domain, and more than 90% of tumor derived missense mutations of human p53 fall within this region. This domain folds in a protease-resistant core, whose structure is coordinated by a Zinc atom. Tetrameric p53 binds DNA recognising the sequence 5'-PuPuPuC(A/T)-3' oriented as four inverted repeats: ⇨ ⇦ ⇨ ⇦. Through an approach based on immune

selection of DNA fragments bound by p53, two groups determined in 1992 the half-site sequence (RRRCWWGYYY) which now is generally considered as the canonical consensus p53 binding site [56, 74]. Most of the genes which have been reported to be transactivated by p53 contain such sequence, even if there is conspicuous variability, both in the base sequence and in the number of nucleotides separating two decameric half-sites. The three dimensional structure of the core domain of human p53 bound to DNA has been resolved in 1994 [26]. Strikingly, the crystal showed that residues R248 and R273, which are the most frequent mutations found in p53, directly contact the phosphate backbone of DNA; this provided an elegant structural explanation for the observed loss of SST activity. Other mutations tend to disrupt the structure of the domain, also impairing the correct protein contacts with DNA and thus abolishing SST. Structural mutations alter the conformation of the core domain, exposing novel epitopes; accordingly, specific monoclonal antibodies such as PAb240 can discriminate between mutant and wt proteins. Mutation of alanine 138 to valine (Ala 135 in the murine protein) confers a temperature-sensitive phenotype to p53 [157, 164]: at 39°C it has a mutant conformation (and is reactive to PAb240); at 32°C is assumes a wild type conformation, is fully functional, and is no longer reactive to PAb240.

The tetramerization domain

As already mentioned, p53 is a tetramer in solution. The domain mediating tetramerization resides between aminoacids 322 and 355 and its three-dimensional structure has been resolved [110]. The p53 tetramer can be envisioned as a dimer of dimers. Two monomers associate in a nearly anti parallel manner and their polypeptide chains are intertwined. Then, two dimers are held together by a large hydrophobic interface. The tetramerization domain is connected to the DNA binding domain by a flexible linker. Within this sequence is located a functional nuclear localisation signal (NLS). Several proteins have been reported to interact with p53 through the oligomerization domain, but contacting different residues than the ones involved in homo-

tetramerization. Very recently it has also been proposed that a functional nuclear export signal (NES) is located within the oligomerization domain (G. Wahl, 9th p53 workshop, 1998). The hydrophobic NES sequence would be buried inside the oligomerization domain in the tetramer, but would be exposed and fully functional in a p53 dimer. This observation adds an intriguing further level of complexity to the regulation of cellular localisation and stability of the p53 protein.

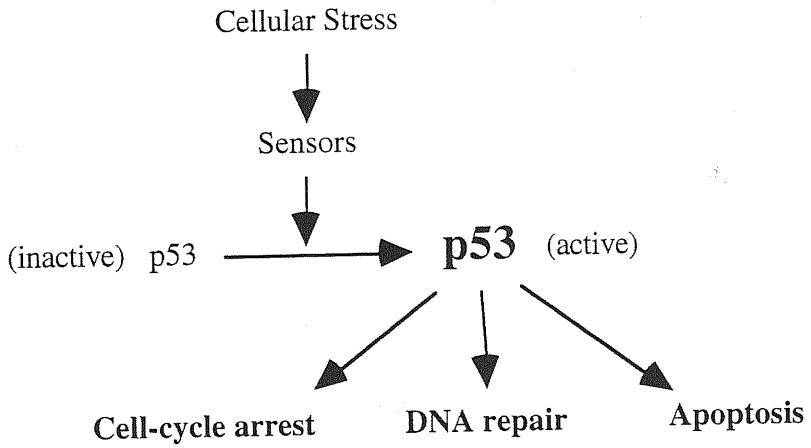
The C-terminal regulatory domain

The last 26 aminoacids at the C-terminus constitute a small protease-sensitive domain rich in positively charged residues, which is believed to mediate non specific binding of p53 to DNA and RNA [130]. This short domain is often considered as part of the tetramerization domain, but I prefer to discuss it as a standalone module, since it plays an important regulatory function. Several evidences indicate that p53 requires a structural change to be activated for sequence-specific binding to DNA; deletion of the C-terminal residues, phosphorylation within the C-terminal domain, or binding of the monoclonal antibody PAb421 to the same domain, all these modifications activate sequence-specific DNA binding of the core domain. A similar activation is also induced when the C-terminus of p53 interacts with single-stranded DNA fragments. Also, two splice variants have been described for murine p53, differing in the last aminoacids at the C-terminus, so that the spliced form is "constitutively activated" for DNA binding [254]. Thus, the basic C-terminal domain is believed to hold the core domain in a conformation which is "inactive" for DNA binding; specific signals could release such sterical block triggering a conformational activation of DNA binding. The small C-terminal domain appears to have a special affinity for DNA ends and internal deletion loops generated by replication errors; therefore, through its non-specific DNA binding activity, it might bring "latent" p53 to DNA lesions and consequently activate p53 for sequence-specific transcriptional activation.

This C-terminal domain was also reported to stimulate reassociation of single-stranded DNA or RNA [179], and at the penultimate residue, Ser 392 in human p53, can be covalently attached the 5.8S ribosomal RNA [69]. This latter modification is believed to be involved in some obscure functions of wt p53 in translational regulation [1].

The current picture of p53. A stress-response device controlling cell-growth and division

If one tries to make all data on p53 to converge into a single model, perhaps the more plausible is that of a "stress-response device". p53 is not required for normal cell proliferation, and it is essentially dispensable during mammalian development [51]. Instead, p53 plays a fundamental role in orchestrating the cellular response to a variety of "insults" which could directly or indirectly affect the integrity of cellular DNA. Such response aims to prevent unrepaired damage from turning into permanent mutations. In doing this, p53 can be considered as component of a signal transduction cascade, where various stress signals are detected by "sensors" triggering p53 "activation". Activated p53 then co-ordinates a "response" by inducing expression of effector genes, by repressing transcription of other genes, or by directly interacting with protein partners [93, 120, 133]. It is worth noting that such response is not stereotyped, but is finely integrated with metabolic and regulatory pathways, thus being largely dependent on the cellular context.



Upstream events triggering p53 activation

The p53 protein is normally present in extremely small quantities in most cells, essentially because of a rapid turnover rate, on the order of minutes. In addition to low concentration, neo-synthesised p53 probably exists in a latent form, inactive for transcription. Under these circumstances, p53 must receive a signal to activate it to function, where "activation" means increased levels of transcriptionally competent p53 within the cell nucleus [133].

The upstream signals that flow to p53 originate from several stressful situations, the best characterised of which is DNA damage. In fact, p53 is activated in response to diverse types of DNA damage, including double strand breaks (DSB) induced by gamma-irradiation or repair intermediates after UV treatment [264]. Exposure of normal adult skin to UV doses which would cause just a mild sunburn, results in accumulation of activated p53 in the cells of the epidermis [91]. It has been hypothesised that even a single DSB would be enough to induce p53 activation [49]. A possible role for cellular protein kinases in signalling to p53 has been proposed. For instance, p53 can be phosphorylated in vitro by DNA-PK, a kinase specifically activated by DSB (see below). Also, p53 response to gamma-irradiation is strongly impaired in cells from Ataxia-Telangiectasia patients, indicating that the ATM protein kinase is involved in activating p53 upon DSB [118]. Notably, AT cells display a normal p53 response to UV treatment and other stress-conditions, demonstrating that multiple pathways exist that activate p53 in response to different stimuli.

Activation of p53 can be observed upon a wide variety of other stressful conditions, including hypoxia [81, 82], disruption of microtubules [235], depletion or unbalance of the cellular pool of ribonucleotides [138], heat or cold shock [181, 182], low pH [183], radioactive labelling [259], addition of calcium-phosphate precipitates [200] and even exposure to space flight conditions [180]. Not all these conditions can be easily correlated, directly or indirectly, to genome-damaging consequences; therefore, it is possible (and very attractive as well) that apart from DNA-damage, p53 monitors other

aspects of cellular "life": for example its metabolism, replicative senescence, or proto-oncogene activation.

If the molecular pathways by which p53 is activated in response to DNA damage are still poorly understood, the pathways by which p53 is activated in response to all these diverse other conditions are almost a "black box".

Anyway, researchers are beginning to understand the biochemical basis of p53 activation which, independently of the inductive signal, consist in essentially two events: 1) the amount of p53 protein within the cell is increased, as a consequence of reduced degradation and perhaps also augmented neosynthesis; 2) latent p53 is conformationally activated by specific post-translational modifications. Since active p53 has to be in the nucleus, regulation of p53 degradation, regulation of its cellular localisation and regulation of its sequence specific DNA binding are tightly correlated, and each of these regulatory steps can be targeted by specific signals.

Regulating protein half-life. The example of the Mdm2-p53 regulatory loop

Activated nuclear p53 induces transcription of several target genes, among which is mdm2 [194]. The p53-induced Mdm2 protein not only interferes with SST by binding to the transactivation domain, but also stimulates p53 degradation by cytoplasmic proteasomes [98, 124]. Indeed, p53-Mdm2 complexes are likely transported out of the nucleus by means of the nuclear export signal (NES) of Mdm2 (see Figure 2) (A.J. Levine, 9th p53 workshop, 1998). Notably, DNA damage induced N-terminal phosphorylation of wt p53 interferes with Mdm2 binding, thus stabilising p53 protein [219]. This p53-Mdm2 auto regulatory feedback loop has a crucial importance, evidenced by the observation that early embryonic lethality of mdm2 nullizygous mice is rescued by deletion of p53 [171]. Thus, Mdm2 plays a key role in keeping p53 under control; in light of this observation, it is not surprising that Mdm2 is overexpressed in many tumors bearing a wt p53 allele [194].

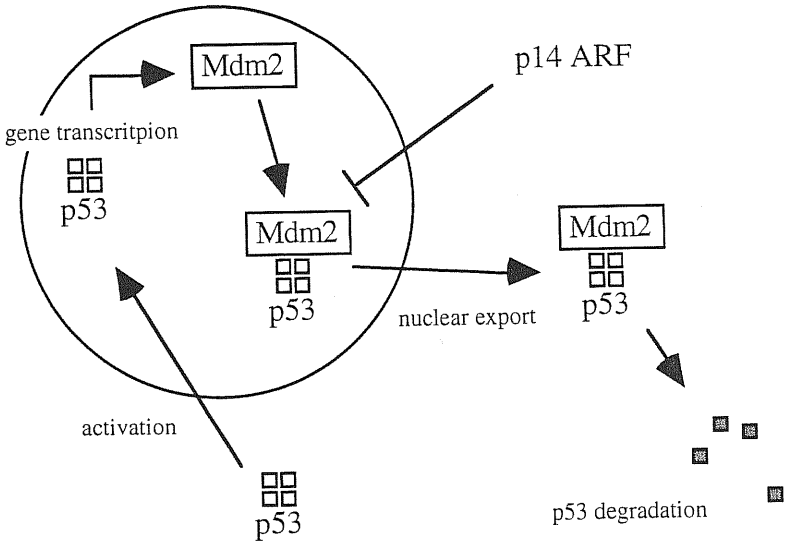


Figure 2
Schematic representation of the Mdm2-p53 regulatory pathway.
Activated, nuclear p53 induces expression of Mdm2, which in turn binds to p53 and mediates its nuclear export and degradation by cytoplasmic proteasomes. p14ARF negatively regulates Mdm2.

Recently, it has been shown that the tumor suppressor protein p14ARF, encoded by the alternative reading frame of the INK4a locus (p19ARF in mouse), is a negative regulator of Mdm2 function [198, 267]. p14ARF physically interacts with Mdm2, but apparently without blocking Mdm2 interaction with p53, thus suggesting the existence of a ternary complex (K. Vousden, 9th p53 workshop, 1998). Strikingly, a correlation between p14ARF induction and oncogene expression has been recently uncovered: it seems that members of the E2F family of transcription factors directly transactivate the alternative promoter of p14ARF, thus increasing its expression [11]. Analogously, p19ARF induction has been observed upon expression of activated Ras in murine cells [192]. These reports suggest a possible regulatory pathway inducing stabilisation of p53 in response to oncogene activation or replicative senescence.

Another possible regulatory pathway influencing p53 stability involves the stress-regulated JNK kinases. It has been shown that non-active JNK kinases, interacting with the N-terminus of p53, can promote its degradation in non-stressed cells [72]. JNK activation upon cellular stress could therefore downmodulate this degradation pathway, thus increasing p53 protein half-life. The importance of the regulation of p53 turnover is clearly underlined by the observation that tumor derived p53 mutants are generally more stable than the wild-type protein, and that the transforming potential of the HPV-E6 oncoprotein correlates with its ability to stimulate p53 degradation.

Conformational activation. Analysis of p53 phosphorylation

About the post translational modifications which are thought to modulate p53 activation, an important role is probably played by phosphorylation. Several phosphorylation sites have been mapped within the N-terminus and the C-terminus of p53 (Figure 3), most of which are evolutionary conserved (for recent review see [120, 161, 162]).

Serine 15 is phosphorylated *in vitro* by DNA dependent protein kinase DNA-PK, a kinase specifically activated by DNA double strand breaks (DSB), and fundamental for DNA repair. Such phosphorylation suggests an appealing link between damage-sensing and p53 activation; nevertheless it seems that phosphorylation on Ser15 is not required for SST, and p53 is efficiently activated by DSB in cells lacking functional DNA-PK [16]. Recent observations suggest that ATM protein kinase is involved in phosphorylating Ser15 upon irradiation: ATM can phosphorylate Ser15 in vitro; ATM kinase activity is greatly increased by IR (but not UV) damage; AT cells show reduced phosphorylation at Ser15 when treated with gamma IR [8, 25]. Notably, phosphorylation at Ser15 correlates with accumulation of p53 protein (reduced degradation rate) and activation of SST activity [219].

Other Ser residues within the first 10 aminoacids in p53 are phosphorylated by the 1α isoform of Casein Kinase (CK1) both in vitro and in vivo. CK1 of yeast and

Drosophila are activated by DNA damage. CK1 in mammals are believed to be involved in controlling DNA replication and mitotic spindle events; yet, the significance of such kinases in regulating p53 function is still essentially undefined [162].

Two residues within the proline-rich domain can be phosphorylated by members of the MAP and SAP families of protein kinases. These kinases are activated in response to several signals such as growth-factors and TPA. Phosphorylation within the poly-proline region might modulate its function as a protein docking domain, but it is still not clear whether this phosphorylation has a functional significance in vivo [161, 162].

At least three phosphorylation events take place at different locations within the C-terminus of p53, and each of these modifications stimulates the sequence-specific DNA binding function of the protein. Serine 315 in human p53 is selectively phosphorylated by S-G2/M specific cyclin dependent kinases such as A/Cdk2 and B/Cdc2, but not by G1 CDKs [247]. Importantly, this phosphorylation not only stimulates DNA binding, but does so in a promoter-dependent manner [247]. Therefore, cell-cycle dependent phosphorylation on Ser315 could modulate cell-cycle specificity for different p53-inducible genes with differential roles at G1 or G2 checkpoints.

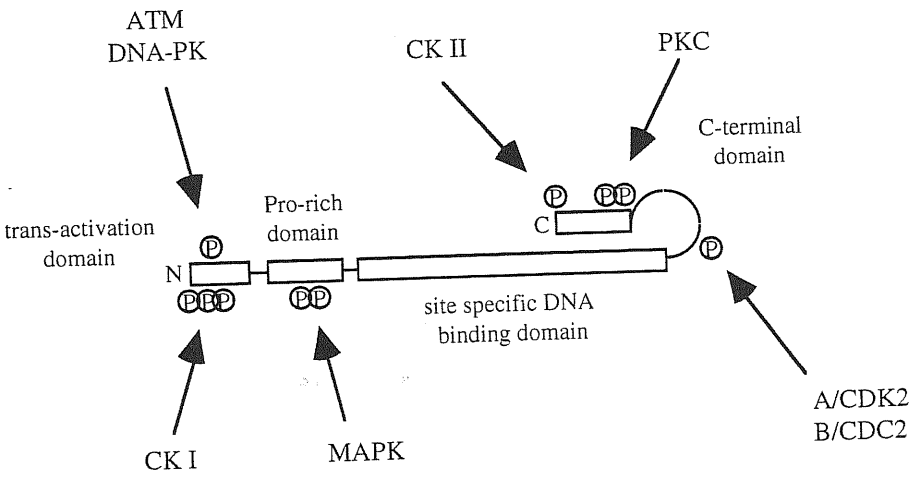


Figure 3
Schematic p53 structure with the C-terminal domain negatively regulating the DNA binding core. Phosphorylation sites are shown, together with the putative protein kinases involved (see text).

p53 is also phosphorylated by protein kinase C (PKC) at several residues in the C-terminal domain. PKC is a family of highly related Ser/Thr kinases involved in several signalling events correlated to growth regulation and apoptosis. The PKC sites on p53 fall within the PAb421 epitope, and PKC phosphorylation dramatically activates DNA binding by p53 [162]. Analogous activation can be obtained by deletion of the last 30 aminoacids, or by interaction with the PAb421 antibody, thus suggesting that phosphorylation can relieve the negative regulatory effect of the C-terminal basic domain.

Finally, p53 is phosphorylated by Casein Kinase II (CKII). The affected residue is the penultimate residue, Ser 392, and also in this case phosphorylation greatly enhances sequence-specific DNA binding, probably by relieving the sterical block imposed by the C-terminal tail. Additionally, this phosphorylation prevents covalent attachment of the 5.8S ribosomal RNA to Ser 392 [69].

In conclusion, specific signals can potentially result in phosphorylation of diverse residues, or single sites can be phosphorylated in response to diverse converging pathways. Furthermore, this regulation might well be cooperative (although there are no experimental evidences yet), cell-type-dependent, and is certainly integrated with the timing of the cell-cycle.

Conformational activation. Other events

The above described observations fit with the model postulating that the C-terminus of p53 contains a flexible regulatory tail which upon phosphorylation induces a conformational shift that activates the core domain for DNA-binding. A recent report demonstrated that the basic C-terminal regulatory domain of p53 can be specifically acetylated by the transcriptional co-activator p300/CBP [83]. Acetylation on lysine residues within this domain could alter its regulatory function by quenching positive charges; accordingly, CBP-dependent acetylation greatly enhances sequence-specific DNA binding by p53 [83]. In addition, it has been proposed that conformational activation could also result from interaction with specific protein partners. Halazonetis

and co-workers recently reported that gamma-irradiation stimulates dephosphorylation of Ser 376 within the PAb421 epitope [249]; this dephosphorylation triggers p53 interaction with 14-3-3 proteins, and such interaction sterically activates the sequence-specific DNA binding function of p53. They could demonstrate that de-phosphorylation and binding to 14-3-3 are dependent on functional ATM, and do not occur in cells from AT patients [249], confirming the functional significance of 14-3-3-mediated conformational activation of p53.

Downstream events mediating p53 function. Biological effects and biochemical activities

The downstream events mediating p53 function as a "guardian of the genome" are multifaceted, but result in essentially two principal outcomes: cell-cycle arrest or apoptosis. Some debate exists on whether p53 is directly involved in DNA repair. These biological effects result from the integration of quite a few diverse biochemical functions of activated wtp53, namely: transcriptional activation, transcriptional repression, re-annealing of single stranded RNA and DNA, direct inhibition of DNA replication, modulation of mRNA translation, 3'-5' exonuclease activity, and specific interaction with selected cellular proteins.

A strong effort has been made to determine the role of each biochemical function in eliciting specific biological effects, to determine which activity of p53 is important for apoptosis, and which is important for cell-cycle arrest, mainly in view of possible therapeutic intervention. Nevertheless, a clear correlation could never be drawn and the current view is that p53 activation is modulated by a number of factors determining, for instance, the balance between transcriptional activation and transcriptional repression activity, selectivity for promoters of different target genes, or sub-cellular localisation of the protein.

Reported properties of wt p53

Biological effects

Induction of G1 and G2 cell-cycle arrest
Regulation of DNA repair (?)
Induction of apoptosis
Inhibition of tumor cell growth

Biochemical functions

Sequence-specific DNA binding
Sequence-specific transcription activation
Repression of transcription
Protein-protein interaction
Reannealing of ssDNA and RNA
Inhibition of DNA replication
3'-5' Exonuclease

Biological effects. Cell-cycle arrest

Perhaps the best characterised consequence of p53 activation is the induction of a G1 cell-cycle arrest. This function is largely dependent on transcriptional induction of p21Waf1, a powerful inhibitor of G1-specific cyclin-dependent kinases (CDK). This protein, also known as Cip1 or Sdi, binds to D/CDK4, E/CDK2, A/CDK2, and perhaps B/CDC2 kinases, and stoichiometrically inhibits their activity [57, 94, 258]. One important function of CDKs is to mediate multisite phosphorylation of pRb (and related proteins) at the G1-S transition (for review see [196, 218]), and inhibition of such kinases by p21Waf1 blocks the cell-cycle [94]. Additionally, p21Waf1 interacts with PCNA, inhibiting its function as a processivity factor in DNA replication [135]. Primary embryonic fibroblasts (MEF) derived from p21-nullizygous mice are partially deficient in their ability to arrest in G1 after DNA damage, confirming the importance of the p21-Rb pathway in p53-dependent cell-cycle arrest [48]. Nevertheless, the analogous phenotype of p53-/- MEFs is far more dramatic [97], and this is generally interpreted as indication that there must be p21-independent pathways contributing to p53-mediated G1 arrest (see

[133]). Also, the issue whether p53-dependent G1 arrest depends essentially on the pRb pathway is not completely assessed; p53-dependent G1 arrest is indeed absent in cells expressing viral oncogenes such as Large T, E1A or E7 proteins, but is still functional in fibroblasts form pRb-nullizygous mice [29, 224]. This paradox can partly be explained by the fact that viral proteins target also other members of the pRb-family, i.e. p107 and p130, which might play a role in p53-dependent G1 arrest [133].

There is also evidence that p53 can induce cell-cycle arrest at the G2/M transition [3, 229]. This effect could be partly ascribed to inhibition of the B/cdc2 kinase by p21Waf1, but it is widely believed that other pathways exist. For instance, the p53-regulated genes Gadd45 and cyclin G both appear to mediate a G2/M arrest at least in certain cell types [220]; and p53 has been shown to upregulate 14-3-3σ, a negative regulator of cdc25c, which is a potential effector of the G2/M checkpoint [104]. Several other evidences for a function of p53 in regulating G2/M events are discussed in more detail in the introductory section of PART 2.

A transactivation-independent function of p53 appears to be involved in mediating cell-cycle arrest upon overexpression of the Gas1 growth-arrest-specific protein [45]. Interestingly, this function of p53 has been demonstrated to require the proline-rich domain of p53, possibly for interaction with some SH3-containing cellular protein transducing the growth-restrictive signal originated from membrane-bound Gas1 [206].

Biological effects. DNA repair?

p53 activation upon DNA damage can arrest cells in G1, thereby preventing replication of damaged DNA. There are however some evidences that p53 might directly regulate processes like DNA repair and DNA replication. It has been reported that purified p53 can inhibit DNA replication *in vitro* [34, 166], and two well characterised p53-inducible genes, Waf1 and Gadd45, specifically interact with PCNA (see [120] for comprehensive discussion). PCNA is a factor involved in both DNA replication and DNA repair and interestingly, p21Waf1 interaction with PCNA inhibits its function in DNA

replication, but leaves untouched its function in DNA repair [135]. p53 also interacts directly with cellular proteins that play a role in DNA-repair, such as RP-A [134], different subunits of TFIIH transcription-repair factor, including XPB and XPD helicases [245, 256], and the CSB (ERCC6) strand-specific repair factor [245]. Furthermore, p53 is known to bind irradiated and/or mismatched DNA [130], and p53 itself has both exonuclease [174] and anti-helicase activity [179]. Nevertheless, these clues are strongly counterbalanced by three important observations: (1) cells from Li-Fraumeni patients, defective for global DNA repair, have normal transcription-coupled repair (the one involving TFIIH) [70]; (2) p53 nullizygous mice display normal rates of DNA repair [108, 212]; (3) p53 apparently does not influence DNA repair *in vitro* [132, 211].

These contradictory arguments still await experimental clarification; the recent discovery of p53 related genes also suggests that functional redundancy might eventually explain at least some of these paradoxes.

Biological effects. Apoptosis

Apoptosis is often referred to as programmed cell-death, or cellular "suicide", and is a complex phenomenon, involving precise biochemical events, which plays a crucial role in development and disease [64, 255]. Since the first reports dated 1992, a large amount of studies have clearly demonstrated that p53 plays a role in triggering programmed cell death under several conditions (reviewed in [12, 93]). Indeed, p53 is required for induction of apoptosis in thymocytes exposed to ionising-radiation [143], and more generally p53 can elicit a dramatic cell-death response when overexpressed in some cell-types [93]. Importantly, this response can be modulated by other factors. For example, activation of a temperature-sensitive p53 transgene in M1 leukaemic cells results in massive apoptosis and no G1 arrest. But if p53 is activated in the presence of IL-6, the same cells enter G1 arrest without cell-death [260]. Thus, specific signalling can reverse p53-mediated apoptosis. It is widely believed that p53-dependent apoptosis is an important mechanism by which transformation is suppressed in oncogene-expressing

cells. Tumor growth and progression are greatly influenced by apoptotic phenomena, and the efficacy of many cancer therapies is correlated to the ability to stimulate a p53-dependent apoptotic response [81, 119, 142]. An elegant study demonstrated the importance of p53-dependent apoptosis in tumor progression [231]. Transgenic mice expressing the SV40 large T antigen, which targets both p53 and pRb family proteins, develop aggressive chorioid plexus tumors; mice bearing a truncated large T, capable of binding Rb but not p53, still develop tumors, but such tumors grow very slowly. This difference was shown to be p53-dependent, and indeed, the same truncated large T could induce very aggressive tumors in p53 knock-out mice. Analysis of tumor samples from p53-/- and p53+/+ mice revealed that the cell proliferation rate was identical in both aggressive and slow tumors. It was instead observed that slow-growing tumors displayed massive apoptosis, and such apoptosis was absent from the aggressive tumors developed by p53-/- mice. It was therefore concluded that functional p53 can suppress tumor development by inducing apoptosis of transformed cells [87].

The biochemical basis of p53-induced cell-death appear to involve both transcriptional dependent and independent functions, and a few p53-inducible genes are good candidates to mediate p53-dependent apoptosis: for example Bax, a Bcl-2 interacting protein with strong pro-apoptotic function [170] or the Fas/APO-1 "death" receptor [191]. p53 can also transcriptionally downregulate expression of the anti-apoptotic gene Bcl2 [169]. Recently, Vogelstein and co-workers isolated a number of transcripts upregulated during p53-dependent apoptosis, which they dubbed PIGs [197]. Several of the transcripts which were induced more than 10-fold, encode proteins that could generate, or respond to oxidative stress [197]. These observations induced the authors to propose a model for p53-dependent apoptosis: activation of p53 causes upregulation of several genes, including oxidoreductases, as an early event. The proteins encoded by those genes, then, increase the content of reactive oxygen species, which in turn damage the mitochondria. Mitochondrial damage stimulates the caspases which activate the apoptotic process [197]. This hypothesis is supported by the observation that

in several models of p53-dependent cell-death, the apoptotic process involves generation of reactive oxygen species. These results are new and very promising, since they provide an explanation for cell-type dependence of the response to p53 activation: the sensitivity of each cell-type to p53-induced apoptosis would depend on the balance between induction of PIGs redox genes, and the capacity of the cell to cope with reactive oxidants. This, of course, is also depending on the levels of other effectors (e.g. bax), acting on different pathways. In line with this multi-factorial picture, overexpression of a single redox gene, PIG3, was not sufficient to induce apoptosis [197]. A recent study demonstrated that the Proline-rich domain is required for binding and transactivation of the PIG3 promoter by p53 [241]. The same work also suggested that the proline-rich region is involved in p53-dependent transcriptional repression [241]. These results support previous studies which correlated transcriptional repression with apoptosis [207, 175], and provide an explanation for previous observations indicating a requirement for the poly-proline domain in p53-dependent apoptosis [208].

Biochemical activities. Transcriptional repression

Several studies demonstrated that p53 can negatively regulate transcription of a number of cellular and viral genes (reviewed in [120]). One important aspect of this activity is probably dependent on the ability to bind TBP (TATA-binding protein) and other components of the basal transcription machinery. It is generally believed that such interaction is productive in the context of a functional p53-binding site, thus activating gene expression, but is repressive in the absence of a p53-responsive element. Accordingly, ectopic expression of p53 represses transcription from TATA-containing promoters such as HSV-TK or SV40 early promoter. The results obtained through overexpression experiments are finding support from differential expression studies. For instance, it has been shown that p53 specifically downregulates expression of the MAP4 microtubule-associated protein [175]. Interestingly, although the MAP4 promoter apparently lacks consensus p53-binding sites, immune-selection experiments suggest that

p53 actually binds to MAP4 promoter (M. Murphy, 9th p53 workshop, 1998). This raises the intriguing possibility that transcriptional repression by p53 depends on cellular co-factors mediating selectivity for binding to "repressional" sites on DNA. In line with this hypothesis, it appears that p53-dependent repression of the hsp70 promoter requires interaction of p53 with the CCAAT-binding transcription factor CBF [120]. A recent study in which gene expression was differentially analysed in tissues from p53+/+ vs. p53-/- mice after exposure to ionising radiation, provided indication that a large number of genes might be influenced by p53 activity, and many of them are repressed [123]. In line with these observations, during a microchip-based approach to study differential expression of cellular genes, at least 70 transcripts, comprising several tubulin isoforms, were found to be specifically downregulated by wt p53 in both human and murine cells (A.J. Levine, 9th p53 workshop, 1998).

Biochemical activities. Protein-protein interactions

p53 can interact with a number of cellular and viral proteins; these are schematically listed in Figure 1. One very important cellular p53-interacting protein is p53 itself. In fact, p53 is a tetramer in the cell (see description of the tetramerization domain). Nevertheless, dimeric p53 efficiently bind to consensus sites *in vitro*, and monomeric variants can be transcriptionally active [233]. Also, a monomeric C-terminally truncated p53 is capable of inducing apoptosis, at least in certain cell types [99]. Also, it has been recently suggested that a nuclear-export signal (NES) is located in the tetramerization domain, mediating nuclear export of p53 monomers and dimers, but not tetramers (G. Wahl, 9th p53 workshop, 1998); thus the oligomerization status of the protein could influence its localisation. Of fundamental importance, wtp53 proteins binding to mutant p53 proteins become functionally inactive. Accordingly, most of the tumor-derived point mutations confer to p53 a dominant negative phenotype, since mutant/wild-type p53 hetero-oligomers are strongly impaired in tumor-suppressing function [92, 167].

Another functionally important p53 interaction is that with the cellular protein Mdm2. In fact, binding of Mdm2 to p53 modulates its SST activity, stability, and nuclear localisation. Indeed, Mdm2 binds p53 on the same residues involved in transcription activation [137], and this blocks the ability of p53 to act as a transcription factor and initiates proteolytic breakdown of the p53 protein. Mdm2-p53 complexes are transported out of the nucleus using the nuclear export signal of Mdm2, (A.J. Levine, 9th p53 workshop, 1998). Mdm2 binding to p53 is finely regulated both by controlling cellular levels of Mdm2 and by N-terminal phosphorylation of p53, which relieves the interaction [219]. Since Mdm2 is upregulated by activated p53, the significance of the Mdm2-p53 interaction is generally considered as a negative feedback loop to constrain p53 activity.

A cellular protein related to Mdm2 has been cloned in a screening for p53-interacting proteins. This protein, named Mdmx, binds p53 at the same residues recognised by Mdm2 and shares some p53-inhibiting properties of Mdm2; Mdmx is ubiquitously expressed and is not regulated by p53 [223].

An interesting p53 interacting protein is the c-Abl protein kinase. Abl is activated upon DNA damage, it binds to p53, and enhances its transcriptional activity. Overexpression of c-Abl inhibits cell growth in a wt p53-dependent manner [78]. Furthermore, Abl is an SH3 containing protein and thus could potentially interact with the Proline-rich domain of p53 [133].

p53 also interacts with the product of the Wilm's tumor gene, WT-1, at least in kidney cell lines [153]. WT-1 is a Zn-finger transcription factor which can both activate and repress transcription. Interaction with WT-1 increases stability of p53, activates its SST activity, and downregulates p53 apoptotic function leaving untouched its cell-cycle arrest function [152]. Binding of WT-1 to p53, modulates the capacity of WT-1 to repress transcription from some cellular promoters, such as EGF-R [63].

Another interesting p53 interacting protein is the product of the tumor suppressor BRCA1, a protein frequently deleted in breast cancer, and functionally important for cellular responses to DNA damage [227]. BRCA1 binds p53 at the oligomerization

domain and increases SST function, acting as a co-activator [190, 266]. It is possible that BRCA1 binding to p53 interferes with tetramerization, since a truncated BRCA1 maintaining the p53-binding domain has a dominant negative effect on p53 function [266].

Among several cellular genes that have been identified as p53-interacting proteins, the p53 binding protein 2 (p53BP2) is particularly interesting. p53BP2 is an SH3 containing protein which interacts with p53 at the core domain. Surprisingly, resolution of the crystal structure of the p53 bound to p53BP2 revealed that the SH3 domain is not interacting with the poly-proline region in p53 [79]. p53BP2 specifically binds to protein phosphatase 1 (PP1), suggesting that it might stimulate p53 dephosphorylation by PP1 [101, 228]. p53BP2 also interacts with Bcl2 [176].

Other cellular proteins that interact with p53 can be grouped in three main categories: 1) General transcription factors, such as components of TFIID, TBP, and components of TFIIH; 2) Transcriptional activators, such as Sp1, CCAAT-binding factor CBF, and WT1; 3) Replication and repair proteins, such as RP-A, TFIIH components, and Cockaine Syndrome protein B (CSB). Such interactions suggest the molecular basis for activities of wt p53 in transcriptional regulation, DNA replication, and DNA repair. The list of p53 interacting proteins is continuously growing, although some of these interactions still have unclear significance in the context of p53 function, and much of these data come from yeast or *in vitro* systems and overexpression experiments (for review see [120]).

p53 interaction with viral proteins

Consistent with the fundamental role of p53 in tumor suppression, a number of oncogenic viruses encode proteins that functionally interact with p53 and hinder its function. These viruses generally target also proteins of the pRB family, and interference with both of these pathways is required for their transforming activity. The large T antigen of SV40 binds and functionally inactivates both p53 and pRb [270]; indeed, p53

was first identified as a cellular protein interacting with large T [127]. High risk human papillomaviruses HPV16 and HPV18 encode the E6 protein, which binds to p53 and mediates its interaction with E6-AP, a cellular protein that stimulates p53 degradation via the ubiquitin pathway [214, 252], and E7, which binds and inactivates pRB [237]. Adenoviruses produce E1A, well known interactor of pRb family-members, and E1B, encoding two peptides that functionally target cellular p53: E1B55K and E1B19K. The E1B 55K protein binds the N-terminal transactivation domain, recognising the same residues involved in transcriptional activation and Mdm2 binding [137]. The E1B 19K protein apparently does not bind to p53, but functionally inhibits the ability of p53 to induce apoptosis; such inhibitory effect correlates with loss of the transcriptional repression function of wt p53 [40, 207]. Several reports indicate that p53 is also bound by the X protein of hepatitis B virus; HBX is a small protein displaying multiple activities and is involved in viral replication, activation of certain signal transduction pathways, and development of hepatocellular carcinomas. Binding of HBX to p53 inhibits its SST activity, and abrogates its apoptotic functions, possibly by sequestering p53 in the cytoplasm [60] and references therein). HBX might interfere with p53-dependent apoptosis by inhibiting p53 transcriptional repression function, analogously to what reported for E1B 19K [60].

Biochemical activities. Sequence-specific transcriptional activation

Perhaps the principal activity of the p53 protein is transcriptional upregulation of a number of cellular genes containing specific p53-responsive elements in their genomic sequences. Several studies have contributed to definition of a consensus sequence specifically recognised by p53 [56, 74, 236]. The canonical consensus for p53 binding contains two copies of the decamer RRRCWWGYYY (R=Purine, W=A or T, Y=Pyrimidine), separated by 0 to 13 base pairs. Each decamer is composed of two pentameric units (RRRCW) arranged as inverted repeats (⇨ ⇦). This distribution is consistent with each p53 molecule in a tetramer binding to a pentameric element.

Definition of the crystal structure of the core domain bound to DNA, revealed that p53 makes contacts with DNA bases both in the major and minor grooves [26]. Within the major groove, two different residues interact with bases 2 and 3 of a pentamer, but the most crucial contact is made with the invariant C:G pair in position 4. Within the minor groove, a contact is made with the A:T pair (position 5); this interaction must be crucial, since the aminoacid involved, Arg248, is one of the most frequent mutations found in human cancer [26].

It is conceivable that flexibility of the domain allows for some variability in the sequence; indeed, analysis of many genes that are regulated by p53 *in vivo* reveals that functional responsive elements often have 'errors' in the first three bases of the pentamer. Functional p53-binding sites might even lack one base, or have one extra base between the highly conserved C(4) and G(7), as in RRRCWGYYY or RRRCWWWGYYY.

Several p53-inducible genes contain a p53-responsive element within an intron, which appears to be a distinctive feature of p53 as a transcription factor. Finally, although a single p53-binding site (made of two perfect consensus decamers) is fully functional in promoter-reporter experiments, a common feature of most p53-regulated cellular genes is that functional responsive elements contain four or more decamers, and often quite divergent from the consensus [17]. Thus, p53 can recognise diverse sequences, and accumulating data suggest that not all binding sites are the same to p53. Of note, a recent study has analysed the binding properties of purified p53 on two functional p53-RE from the human WAF1 promoter. The two sites differ for the presence of a G in place of the C(4) of a pentamer, and display opposite properties: the "canonical" site is bound more efficiently by p53 in complex with pAb421. The "variant" site is bound by p53 alone, but not by p53-pAb421 complexes. Since it is believed that binding of pAb421 to the C-terminal domain triggers conformational activation of p53, the differential selectivity of the two p53-REs might be functionally relevant. Another example of p53-RE selectivity comes from a study in which sequence specific DNA binding by purified recombinant p53 was tested after incubation with different CDKs; phosphorylation by S/G2-specific

CDKs increased p53 binding to the RE of Gadd45 and Waf1, but not to RE of RGC, or MCK [247]. Selectivity for binding to the promoters of certain genes rather than others, can therefore be modulated by post-translational modifications or protein-protein interactions. As already described in this introduction, p53 binds TBP and other components of TFIID and TFIIH; accordingly, at least in certain promoter sequences, p53 binding is sufficient to nucleate assembly of the pre-initiation complex and stimulate transcription, even in the absence of a proper minimal promoter [39].

An overview of published p53-inducible genes

During the past years, ever since it became clear that p53 is a transcription factor, a significant number of genes regulated in a wt p53 dependent manner has been identified, and the list is continuously growing. In a recent review, Ko and Prives tried to establish some criteria for defining a 'proper' p53 responsive gene [120]: 1) The gene must contain binding sites that can be specifically recognised by wt p53. 2) These sites must be capable of mediating wt p53-dependent expression of a reporter gene. 3) These sites must display p53-responsiveness in the context of the genomic promoter. 4) The gene should be induced in a wt p53-dependent manner in cells exposed to stress conditions (e.g. DNA damage) that activate endogenous p53 function. Up to now, only few of the genes described as p53 targets actually fulfil the criteria indicated above. In reviewing the growing list of accredited and putative p53-regulated genes, I shall group them according to different aspects of p53 biological activity.

Genes involved in cell-cycle regulation

As already mentioned in this introduction, it is unanimously believed that the principal function of wt p53 is that of sensing real or potential DNA damage and in consequence halting cell division. Thus, p53 participates in a DNA damage checkpoint presumably active at all phases of the cell cycle. Checkpoint functions of p53 appear to

require its transactivation activity and, at least in some cases, cell cycle arrest in response to DNA damage is mediated by specific gene products upregulated by p53.

p21WAF1/CIP1/SDI1

WAF1 is one of the best characterised p53 responsive genes [57, 94]. Its encoded 21kDa protein is normally found in quaternary complexes with cyclin/CDKs together with the DNA polymerase processivity factor PCNA. p21 can bind to D/Cdk4, E/Cdk2, A/Cdk2, A/Cdc2 and B/Cdc2 kinases. Although p21Waf1 is a normal component of such complexes, increased levels of p21 have a strong inhibitory effect on these CDKs. Since activity of these kinases is instrumental for the G1/S transition, high levels of p21Waf1 efficiently block progression through the cell-cycle and cause G1 arrest [258]. Furthermore, p21Waf1 binding to PCNA inhibits its activity in DNA replication, possibly leaving untouched its activity in DNA repair [135]. Two separate functional p53-RE are contained in the human WAF1 promoter, approximately 3 Kb and 2 Kb upstream from the transcription start site, and Waf1 is efficiently induced by wt p53 in a multitude of experimental systems. Thus p21Waf1 is undoubtedly one important mediator of p53-induced G1 arrest [54]. Nevertheless, fibroblasts from WAF1-nullizygous mice are only partially deficient in cell-cycle arrest after DNA damage, indicative that there are alternative pathways for p53-induced cell-cycle arrest [20, 48]. p21Waf1 protein can be accumulated upon DNA damage in murine fibroblasts lacking functional p53, and is cell-cycle regulated. p21Waf1 is also regulated by replicative senescence and plays a role in differentiation of several cell-lines [136, 149, 165]. A possible role for p21Waf1 in regulating G2/M transition was also recently proposed, since p21 protein binds to A/Cdc2 and B/Cdc2 kinases and undergoes nuclear re-accumulation at the onset of mitosis [55].

PCNA

Controversial evidences indicate the Proliferating Cell Nuclear Antigen as being upregulated or downregulated by p53. PCNA is a DNA-damage inducible auxiliary factor for DNA polymerases δ and ε, and plays a fundamental role in DNA replication and repair. A functional p53-RE is present in the promoter of human PCNA; this sequence is bound by wt p53 *in vitro*, and this element confers p53-dependent regulation to a reporter gene in transfection experiments [173, 222]. Nevertheless, in a human glioblastoma cell-line expressing a regulatable p53 allele, PCNA was shown to be downregulated during p53-mediated growth-arrest [163], although this repression could just be a consequence of reduced S-phase entry. A possible explanation for this apparent paradox is that the cellular levels of activated p53 influence the effect on PCNA promoter: low levels of p53 would transactivate PCNA promoter, high levels of p53 would repress it. Anyway, data unambiguously demonstrating p53-dependent upregulation of PCNA *in vivo* are missing. Interaction of p21Waf1 with PCNA inhibits its function as a processivity factor in DNA replication, but not its role in DNA repair [135]; it is thus conceivable that a p53-dependent induction of PCNA might help in modulating DNA repair.

Gadd45

Gadd45 (Growth-arrest and DNA damage induced) belongs to a group of transcripts upregulated by growth-arrest and several stressful conditions [71]. Gadd45 is induced after DNA damage by both p53-dependent and independent pathways. Gadd45 encodes a nuclear protein that interacts with PCNA and inhibits entry into S-phase, thus suggesting that it might be part of a pathway alternative to WAF1 in the cellular response to DNA damage [114, 226]. A functional p53-RE is located within the third intron in both human and hamster GADD45 genes [114]. Cells expressing reduced levels of Gadd45 display deficient G2/M arrest upon UV treatment, although they still can arrest at G2/M upon gamma-irradiation.

This evidence suggests that Gadd45 might be a G2/M checkpoint gene. Overexpression of Gadd45 in primary human fibroblasts caused accumulation of cells with a 4N DNA content and a mitotic phenotype (i.e. positive to the MPM-2 antibody). This cell-cycle arrest can be recovered by overexpression of cyclin B1 or cdc25, suggesting that Gadd45-mediated G2/M arrest occurs by down-modulation of MPF activity (C.C. Harris, 9th p53 workshop, 1998).

Cyclin G

The product of the cyclin G gene is a novel cyclin-like protein whose function is essentially unknown. Its levels are not regulated during the cell cycle and it does not complex with any known CDK. It was isolated through immune-selection of genomic sequences bound by p53 in rat cells [262]; it contains two functional p53 binding sites, one in the promoter and one in the first intron, and is promptly induced by DNA damage [184]. Although p53-dependent regulation of a cyclin gene suggests a further correlation with the cell-cycle machinery, at the moment there are no clear data on a possible role of cyclin G in cell-cycle progression. Overexpression of cyclin G in human carcinoma cells stimulated proliferation, and high levels of cyclin G can be found in a subset of human osteosarcomas [225]. In REF exposed to DNA damaging drugs such as doxorubicin, cyclin G accumulation correlated with G2/M cell-cycle arrest; antisense oligonucleotides to cyclin G inhibited such arrest, thus suggesting that cyclin G might play a role in the G2/M checkpoint [220].

RB

A functional p53 binding site has been described in the human Retinoblastoma gene (RB1) promoter [189]. Although this site can confer p53-dependent regulation to a reporter gene, and wt p53 binds to it *in vitro*, there are no evidences that pRb expression is increased upon physiological activation of p53 in human cells.

14-3-3σ

The sigma member of the 14-3-3 family of proteins was identified as a p53 inducible gene through a SAGE approach in a human colon cancer cell line undergoing p53-mediated G2/M cell-cycle arrest [104]. Two functional p53-RE are present in the genomic sequences of human 14-3-3σ and the protein is induced by DNA damage in a wt p53-dependent manner. Interestingly, 14-3-3σ binds to Cdc25C phosphatase, and prevents it from activating the B/cdc2 kinase (MPF) at G2/M transition; as a consequence, overexpression of 14-3-3σ delays G2->M transition [104]. Although 14-3-3 proteins are generally abundant in the cell, p53-dependent upregulation of a negative regulator of MPF activation suggests a molecular mechanism for the postulated functions of wt p53 at the G2/M checkpoint.

CDC25C

Through a computer-based approach, a putative p53-binding sequence was identified in the promoter of human cdc25c protein phosphatase. This site is not a perfect consensus, but is related to one of the functional p53-REs found in the promoter of human WAF1 [202]. Purified p53 can bind to a synthetic oligonucleotide corresponding to this sequence, and such oligo can confer p53-dependent expression to a reporter gene. At the moment there are no evidences for p53-dependent regulation of cdc25c *in vivo* and this prediction, therefore, still awaits experimental confirmation.

Genes involved in apoptosis

One of the ways by which p53 can protect the integrity of the genome is the elimination of cells that have suffered excessive DNA damage through induction of apoptosis. The pro-apoptotic function of p53 involves both transcriptional dependent and independent activities. Some promising candidate mediators of transcriptional-dependent pro-apoptotic functions of wt p53 are described below.

BAX

Bax gene is a well characterised transcriptional target of p53 and is induced in response to irradiation in a p53 dependent manner [170]. Bax is a member of the Bcl-2 family of apoptotic regulators, and heterodimerises with Bcl-2. The balance of these two gene products is crucial to the regulation of the apoptotic process: Bcl-2 protecting from apoptosis, and Bax stimulating cell death [2]. Although p53-dependent apoptosis can also occur via Bax-independent pathways, at least in certain cell systems, this gene is undoubtedly an important mediator of p53 apoptotic function.

PAG608

PAG608 encodes a novel putative Zn-finger protein that was cloned as a gene upregulated in murine myeloid leukaemia M1 cells undergoing p53-dependent apoptosis. It is also induced in a wt p53-dependent manner in cells from thymuses of mice exposed to gamma-irradiation. PAG608 protein localises to the nucleus, and induces a clear apoptotic phenotype when overexpressed in several transformed human cell lines [109]. No functional p53-responsive elements have been reported for this gene.

SIAH

In a differential screening to isolate genes upregulated in murine M1 myeloid leukaemia cells undergoing p53-dependent apoptosis, several transcripts were identified that are increased within the first hours of p53 induction [4]. Among these, three cDNAs

were described having significant homology to known genes. One is homologous to rat Phospholipase C ß4, a protein involved in signal transduction from tyrosine kinase receptors. One is homologous to a Zn-finger protein, ZFM1, that was mapped to the multiple endocrine dysplasia locus (MEN1). The third is the murine homologue of *Drosophila* Seven in Absentia (sina) gene, named siah. *sina* encodes a nuclear Zn-finger protein involved in cell fate specification in the developing eye; induction of siah by p53 suggests a link between developmental and apoptotic pathways. Interestingly, growth-suppressive properties of murine Siah are abrogated by expression of BAG1, an anti-apoptotic protein physically interacting with Siah-1A [160]. No functional p53-responsive elements have been reported for this gene.

KILLER/DR5

Killer/DR5 was isolated in a subtractive hybridisation screening for genes selectively expressed during doxorubcin-induced apoptosis of a chemiosensitive ovarian carcinoma cell-line. It encodes a death domain-containing receptor for TRAIL, and is induced by DNA damage in a p53-dependent manner. Its overexpression is growth-suppressive and leads to apoptotic cell death of cancer cells [253]. No functional p53-responsive elements have been reported for this gene.

FAS/APO-1

The cell surface FAS/APO-1 protein is a member of the TNF receptor superfamily and can trigger apoptosis in several normal and transformed cells upon binding of a specific ligand or a monoclonal antibody. Expression of FAS has been shown to be upregulated by wt p53 in several human transformed cell lines [191]. No functional p53-responsive elements have been reported for this gene.

EI24

The EI24 gene was isolated during a differential screening for genes upregulated in murine fibroblasts after induction of apoptosis by Etoposide treatment. It was subsequently shown that Etoposide induction of EI24 requires wt p53, and that EI24 is upregulated when p53 is ectopically expressed in murine fibroblasts. EI24 encodes a novel peptide that has a significant similarity with *C.elegans* CELF37C12.2, a sequenced ORF of unknown function [131]. There are no evidences that EI24 might be a mediator of p53 apoptotic functions, but its upregulation in apoptosis is strictly dependent on wt p53. No functional p53-responsive elements have been reported for this gene, but a human EI24 homologue is upregulated in colon cancer cells undergoing p53-induced apoptosis (see below).

PIGs

The acrostic PIG (for P53 Induced Genes) indicates a group of genes that have been recently identified by SAGE (Simultaneous Analysis of Gene Expression, a technique for quantitative evaluation of cellular mRNA populations) in a human colon cancer cell line undergoing p53-dependent apoptosis [197]. The authors focused on a number of transcripts expressed at more than 10-fold greater levels in p53-expressing than in control cells (the list of such genes is available through the World Wide Web at the site http://welchlink.welch.jhu.edu/~molgen-g/P53-SAGE.HTML). The striking result of this screening is that most of the PIGs corresponded to proteins with activities related to the redox status of the cell. One of these was the human homologue of murine EI24, a transcript induced in a wt p53-dependent manner by etoposide, a drug known to generate reactive oxygen species (ROS) [131]. Other PIGs correspond to genes known to be induced by ROS, or oxidative stress, or correspond to genes directly involved in generating ROS or stimulating superoxide production [197]. PIG3 encodes a protein highly related to TED2, a plant oxidoreductase involved in apoptotic processes during meristems formation. The most similar mammalian gene is a NADPH-quinone

oxidoreductase, which is a potent generator of ROS. The human PIG3 gene has been characterised, and a functional p53-responsive element has been identified in its promoter [197]. Preliminary experiments indicated that PIG3 alone does not induce apoptosis when overexpressed in colon cancer cells; the consequent model emerging from this study implicates that coordinated induction of pro-oxidative enzymes results in an increase of ROS in the cell, which in turn cause mitochondria damage and apoptosis.

Genes involved in growth regulation

A complex and multifaceted aspect of p53 biology is its growth-suppressive effect on normal and transformed cells. p53 has an overall growth-restrictive effect on cell proliferation, and this effect can be exerted through different biochemical pathways, being largely influenced by the genetic background of the cells. Also in this case, genes specifically regulated by p53 should play a fundamental role in modulating such growth-regulatory response.

MDM-2

The mdm-2 gene is amplified in as many as 40% of human sarcomas: it encodes a protein that complexes with p53 and inhibits its activity. Mdm-2 is strongly regulated by p53 and is induced by DNA damage in wt p53 expressing cells [10]. Mdm2 participates in a p53 autoregulatory feedback loop important for cell-growth and animal development, as described previously in this introduction. Mdm2 inhibits p53 SST activity and promotes p53 degradation through the proteasome pathway [98, 124]. Human MDM-2 was shown to interact with E2F/DP1 and with pRB, and thereby stimulate E2F transcriptional activity and repress pRB dependent growth suppression [156, 257]. High levels of Mdm-2 protein block p53 function and possibly Rb function, and are therefore transforming. Murine MDM-2 contains a functional p53-RE within the first intron; upon p53 activation, the gene is preferentially transcribed from an alternative start-site located downstream the p53-binding site, to generate a shorter mRNA starting with exon 2. The

longer transcript, starting with exon 1, is not significantly induced by wt p53 [9]. The first ATG codon is contained in exon 3, but a shorter Mdm2 protein which does not bind to p53 can be synthesised from an internal ATG codon. On the basis of *in vitro* translation experiments, it was suggested that mRNAs transcribed from the first promoter (p53-independent) are preferentially translated from the internal ATG, while mRNAs transcribed from the second promoter (wt p53-dependent) are preferentially translated from the first ATG [9]. Thus, basal transcription from the first promoter might produce a truncated Mdm2 protein with functions not related to p53 (but perhaps linked to the E2F-pRB axis), while p53-mediated transcription from the second promoter might generate high levels of full-length, p53-binding, Mdm2 protein involved in negative regulation of p53 activity.

p53

Transfection experiments suggested that p53 might transactivate its own promoter in murine cells [41], and indeed a sequence responsible for p53-responsiveness was mapped by deletion analysis in the 5' untranslated region of murine p53. This element, although functional in reporter experiments, is quite divergent from the consensus p53-BS and does not bind p53 *in vitro* [41].

EGF-R

The promoter of the human EGF receptor (EGFR) contains a functional p53 responsive element which is efficiently transactivated by both wild-type and mutant p53 in reporter experiments in transfected cells [147]. In response to UV irradiation the kinase activity of EGF-receptors is activated, and such activation might be part of a proliferative response required for replacement of genetically damaged cells. Nevertheless, up to now the data supporting wt p53-dependent induction of the human EGFR under physiological conditions are very weak.

DDR

Discoidin Domain Receptor is the human homologue of rat Ptk-3, a membrane associated receptor tyrosine kinase that was cloned as a gene induced by ionising radiation in rat astrocytes. Analysis of the human DDR gene revealed a consensus p53-BS within the promoter at -1540 bp relative to the translation start site, and accordingly, DDR mRNA is upregulated upon adenovirus-mediated expression of p53 in human cells [209]. Function and physiological ligand of DDR are still unknown.

TGF-α

The promoter of human Transforming Growth Factor alfa (TGF-α) contains two consensus p53-binding sites, and TGF-a expression was induced by wt p53 in a human glioma cell-line expressing a regulatable p53 allele [221]. Deletion analysis confirmed that the proximal site located at -50 bp relative to the transcription start-site is a fully functional p53-RE. The possibility that wt p53 induces expression of TGF-α, together with EGFR and DDR (described above), suggests a dual role for the p53 tumor suppressor in the response to DNA damage: mediating a growth-restraining response to eliminate genetically damaged cells, and activating a proliferative response necessary for their replacement.

CGR11 and CGR19

These are two independent clones isolated during a differential screening in a rat embryo fibroblasts (REF) cell-line stably expressing a ts p53 allele [150]. Both proteins, whose function is still unknown, efficiently suppressed growth of several human carcinoma cell lines in a colony formation assay [150]. Cgr11 protein has two EF-hand motifs, possibly indicating Ca-binding domains; the EF-motifs are required for its growth-suppressing function. No functional p53-responsive elements have been reported for these genes, and no data are available on p53-dependent expression of the encoded proteins.

IGF-BP3

The Insulin-like Growth Factor-Binding Protein 3 (IGF-BP3) is a secreted polypeptide that inhibits mitogenic signalling by IGF-1. Expression of IGF-BP3 is increased by wt p53 in cell lines carrying a regulatable p53 allele, and two functional p53-RE are located in the first and second introns [22]. Given the important function of IGFs in signalling cell-survival, p53-dependent secretion of IGF-BP3 might have both anti-mitogenic and pro-apoptotic activity. IGF-BP3 might therefore be an important mediator of growth-suppressing functions of wt p53. In line with this hypothesis, wt p53 was shown to repress transcription of the human IGF receptor, while tumor derived p53-mutants actually stimulate IGF-R expression [251]

WIP-1

Wip-1 is a novel human protein with significant homology to type 2C protein phosphatase (PP2C). Expression of Wip-1 is induced by ionising-radiation in a wt p53-dependent manner in several human and murine cell-lines, and Wip-1 mRNA is upregulated at the permissive temperature in cells bearing a ts p53 allele. Wip-1 localises to the nucleus and efficiently inhibits growth of human transformed cell lines in colony-formation assays [68]. No functional p53-responsive elements have been reported for this gene.

HIC-1

The HIC-1 (Hypermethylated In Cancer) gene was isolated as a candidate tumor suppressor gene on chromosome 17p, within a CpG-rich island subject to aberrant hypermethylation or allelic loss in several human tumors [243]. A consensus p53 binding site is present in the upstream genomic sequences, and HIC-1 expression is upregulated by wt p53 in a human colon cancer cell line. The HIC1 encoded protein contains an N-terminal Zn-finger domain (Zin), and might function as a transcriptional repressor. HIC-1 significantly suppresses growth and viability of transfected human tumor cell lines [243].

BTG2

BTG2 is highly related to BTG1, a human gene translocated in a B-cell chronic lymphocytic leukaemia and member of a family of relatively small labile polypeptides involved in growth-control [205]. The 5' untranslated region of the BTG2 gene contains a functional p53 binding element, BTG2 is upregulated by transfection of wt p53 in several cell lines, and BTG2 expression is induced by DNA damage in a p53-dependent manner [204]. BTG2 suppresses cell growth in colony formation assays and Embryonic Stem cells nullizygous for BTG2 display a defective response to Adryamicin treatment, suggesting a role for BTG2 in mediating G2/M arrest upon DNA damage [204].

A28-RGS14

The p53-induced transcript A28 was isolated during a differential screening in a cell line expressing an inducible p53 gene; induction of A28 mRNA was also observed in wt-p53 containing human cells exposed to genotoxic drugs [21]. Its encoded protein belongs to a family of GTP-ase stimulating proteins named RGS, which negatively regulate G-protein coupled mitogenic signalling [13, 263]. Overexpression of RGS14 efficiently inhibits activation of the mitogen-activated protein kinase (MAPK) signalling cascade, involved in cell proliferation and transformation [23]. No functional p53-responsive elements have been reported for this gene, and no data are available on p53-dependent expression of the corresponding protein.

Wig-1

Wig-1 was isolated through a PCR-based differential display analysis in a murine lymphoma cell-line bearing a ts p53 allele [246]. Wig-1 mRNA is also regulated by DNA damage in a wt p53-dependent manner. It encodes a protein predicted to have three Zn-finger motifs and a putative nuclear localisation signal, homologous to a Zn-finger protein of *C.elegans* [240]. No functional p53-responsive elements have been reported for this

gene, and no data are available on subcellular localisation and p53-dependent expression of the Wig-1 protein.

GML

The GPI-linked Molecule-Like (GML) gene was isolated during a search for human genomic clones containing p53-tagged sites that had been previously identified through a yeast genetic screening [236]. It therefore bears a functional p53-binding site in the vicinity of its genomic sequences, although this site was not tested for transactivation of a reporter gene. GML encodes a protein sharing significant similarity to GPI-anchored molecules. Its mRNA is upregulated by wt p53, and GML shows moderate growth-suppressing activity in colony formation assays. Expression of GML correlates with increased sensitivity of oesophageal cancer cell lines to Bleomycin treatment [76].

H-Ras

A putative p53-responsive element has been found within the first intron in the human c-H-Ras gene, before the ATG-containing exon. This site functions as a transcriptional enhancer and is bound by p53 *in vitro*. Gel retardation studies employing nuclear extracts from human endometrial and ovarian tumors suggest a possible correlation between elevated p53 levels and increased expression of Ras p21 protein. This correlation might have some significance in the development of gynecological neoplasias [261]. A cDNA tag corresponding to Ras was found to be highly expressed upon activation of wt p53 in REFs [151].

p22/PRG1

The p22/PRG1 gene (PACAP Response Gene 1) was identified as an early response transcript induced in rat pancreatic cells growth-stimulated by PACAP, a peptide activating Adenylate cyclase [213]. A functional p53-RE was mapped in the promoter of rat PRG1, and PRG1 mRNA was induced by activation of a ts p53 allele in REF cells,

and in rat thymocytes undergoing p53-dependent apoptosis after gamma-irradiation [213]. p22/PRG was also transcriptionally induced in human HeLa cells undergoing doxorubicin-induced apoptosis. There are no available data on the regulation of p22/PRG1 at the protein level.

EGR-1

The Early Growth-Response EGR-1 gene encodes a transcription factor induced by serum and a variety of other growth-regulatory stimuli. EGR-1 regulates gene transcription and binds DNA on the same elements bound by WT-1, thus suggesting a possible interplay between the two factors. A cDNA tag corresponding to EGR-1 was found to be strongly upregulated by p53 activation during a SAGE analysis in rat embryo fibroblasts [151]. No functional p53-responsive elements have been reported for this gene.

SGK

sgk is a novel serine/threonine protein kinase which is transcriptionally regulated by growth-factors and glucocorticoids in mammary epithelial cells. Analysis of rat sgk promoter revealed the presence of putative p53-binding sites within 1.5 Kb upstream to the transcription start site. One of these sites was shown to be a functional p53-RE and to be regulatable by p53 in a cell-type dependent manner: sgk promoter was strongly stimulated by p53 in rat mammary cells, but was repressed in fibroblasts [154]. At the moment, there are no clear evidences for p53-dependent expression of endogenous sgk mRNA or protein under physiological conditions.

KAI1

KAI1 is a tumor metastasis suppressor gene encoding a protein with four putative transmembrane domains, highly conserved and widely expressed in mammals. KAI1 inhibits the metastatic process in animal models, and KAI1 expression is down-regulated during tumor progression of several human cancers [52]. A functional p53-RE is located in the promoter of human KAI1 and accordingly, KAI1 transcription is induced by wt p53 [158]. Analysis of human prostatic tumors revealed a significant correlation between KAI1 expression and p53 levels, and loss of the two markers could be statistically associated to poor survival [158].

Secreted growth inhibitors

A recent study was conducted to analyse gene expression in different tissues of p53-wt and p53-null mice before and after exposure to whole body ionising-radiation. This analysis provided a number of cellular genes whose transcription is either induced or repressed in a p53-dependent manner. By computer search of the available genomic sequences, the authors could also find putative consensus p53-binding sites within most of these genes [123]. A number of secreted growth-inhibitory proteins were found to be transcriptionally upregulated by DNA damage in a wt p53-dependent manner, including TGF-β, inhibin-β, several serine-protease inhibitors, and IGF-BP3. In line with this observation, conditioned medium from IR-treated cells could efficiently repress growth of undamaged transformed cells. This observation suggests an interesting "bystander effect" model, postulating that responsiveness of tumor cells to chemotherapy might be potentiated by anti-cancer drugs activating a p53-dependent response in the neighbouring non-cancerous tissue [123].

Genes involved in regulation of metabolic processes

Although less studied, and largely obscure, an involvement of p53 in the regulation of processes that are not directly correlated to growth-arrest or apoptosis is undoubtedly likely. In fact p53 can be activated in response to a variety of stress conditions that are not strictly connected with DNA damage, such as hypoxia, heat/cold shock, or unbalance in the cellular pool of ribonucleotides. Indeed, growing evidences suggest a link between p53 function and angiogenesis, sensitivity to low oxygen, detoxifying systems, and energy metabolism.

TSP-1

Thrombospondin-1 is a large, secreted, matrix glycoprotein which is a potent inhibitor of angiogenesis. TSP-1 mRNA and protein are upregulated by expression of wt p53 in p53 null Li-Fraumeni fibroblasts and the human TSP-1 gene promoter can confer p53 dependent regulation to a heterologous reporter gene in transfection experiments [37]. Inhibition of angiogenesis might be an important aspect of p53 function in tumor suppression; indeed, cells in the hypoxic regions inside solid tumors undergo extensive apoptosis, at least partly p53-dependent, and are subject to a strong selective pressure for acquiring p53 mutations [81].

BAI-1

The BAI1 (Brain-specific Angiogenesis Inhibitor) gene was isolated during a search for human genomic clones containing p53-tagged sites that had been previously identified through a yeast genetic screening [236]. It carries a functional p53-binding site in the vicinity of its genomic sequences, although this site was not tested for transactivation of a reporter gene. Expression of BAI1 mRNA is brain-specific, and could be induced by transfection of wt p53 in a human glioblastoma cell line [177]. No data are available on the regulation of BAI1 protein under the same conditions. BAI1 has the characteristics of a multi-spanning membrane protein with an extracellular domain bearing TSP-type 1

repeats, suggesting a possible role in inhibiting angiogenesis. Accordingly, a recombinant protein containing the TSP repeats of BAI1 could inhibit *in vivo* neovascularization induced by FGF in rat cornea [177]. This observation, together with p53-dependent induction of Thrombospondin-1 (see above), further suggests a link between p53 function and negative regulation of angiogenesis.

MCK

The promoter of muscle Creatine Kinase (MCK) is perhaps one of the best characterised p53-responsive DNA elements [250]. It confers strong p53-dependent regulation to heterologous reporter genes, and contains several functional p53-binding sites [268, 269]. Despite the well characterised p53-responsiveness of its promoter, evidences that Mck mRNA or protein are upregulated by p53 *in vivo* are still missing. If confirmed, p53-dependent upregulation of MCK, a protein involved in homeostasis of ATP, could be envisaged as being part of a control over the metabolism of the p53-arrested cell, perhaps in response to specific energy requirements during the process of DNA repair.

mEH and SM20

Microsomal Hepoxide Hydrolase (mEH) and SM20, a transcript induced in vascular smooth muscle cells upon PDGF-A treatment, were both cloned by differential display in a rat cell line bearing a ts p53 allele [150]. No functional p53-RE have been described for these two genes, and no data are available on the regulation of the encoded proteins. The function of SM20 is essentially unknown, while mEH is an enzyme involved in catalytic detoxification of xenobiotics, including metabolising reactive epoxides which are potential carcinogens.

Smooth muscle α-actin

Smooth muscle (sm) α-actin is transcriptionally regulated during cell proliferation and transformation, being increased at growth-arrest in fibroblasts and smooth muscle cells. α-actin was found to be transcriptionally induced in REF upon activation of a ts p53 allele, concomitantly to increase of actin filament bundles [32]. A functional p53-RE was identified in the promoter of human α-actin and its mRNA was upregulated by DNA damage in human cells [32]. This observation suggests a link between p53 function and formation of well organised microfilament structures typical of arrested cells.

Type IV Collagenase

Through computer analysis of the GenBank database, a consensus p53-BS was identified in the promoter of human type IV collagenase (Gelatinase A, or Matrix Metalloproteinase-2) [15]. Experiments designed to test a possible regulation of this promoter by p53 demonstrated that such site is bound by wt p53 and mediates p53-dependent expression of an heterologous reporter gene. Endogenous type IV collagenase mRNA is induced by DNA damage in a p53-dependent manner [15]. This observation suggests an interesting correlation between p53 function and wound-healing, angiogenesis, and tumor cell invasion. No data were provided on the regulation of type IV collagenase protein.

Other candidate genes for transactivation by p53

The characterisation of a consensus motif for p53 binding and the comparative analysis of many sequences that are functional *in vivo* as p53 responsive elements allows a rather stringent definition of a p53 binding site, and therefore of a putative p53 target gene. During a computer based analysis of genomic sequences available in the databases, a number of potential p53-responsive genes have been identified [17]. Such theoretical predictions obviously await the experimental confirmation, but it is interesting to note that several genes involved in regulation of energy metabolism are included in the list. Apart

from cytosolic Adenylate Kinase (see PART3) and muscle/brain Creatine Kinase (see previous pages), for which there are experimental evidences for regulation by p53, the above described computer search suggested as putative p53-regulated genes also M1 and M2 types of pyruvate kinase, phosphoenolpyruvate carboxykinase, phosphorylase kinase gamma subunit, pyruvate dehydrogenase and M-type 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase [17].

Searching for more p53-regulated genes

The present list of cellular genes induced by p53 indicates that the targets of wt p53 might indeed be very numerous. It also raises legitimate perplexities about the biological significance of regulations that are observed exclusively in cell-culture, under extreme conditions, or with overexpressed p53.

Novel strategies are required to gain a wider knowledge on p53 function, especially in relation to the whole cell function. Promising in this regard are functional genomics approaches employing novel technologies such as cDNA micro-arrays or DNA-chips to compare gene regulation in cells or tissues before and after activation of endogenous p53 function. A recent study in which oligonucleotide DNA-chips were used to detect genes regulated by p53 in human colon-cancer cell lines provided a list of approximately 80 cDNAs that are upregulated, and approximately 70 cDNAs that are repressed by p53 (A.J. Levine., 9th p53 workshop, 1998). This sort of approaches, coupled to systematic cloning and sequencing of all expressed genes, will eventually provide a global picture of how cellular gene expression is regulated upon p53 activation. This will allow researchers to study each gene within the "real-life" scenario of multiple functional interactions with other gene products, inside any given cellular context. Nevertheless, to gain insight into any complex picture it is also necessary to analyse in detail each element by itself, and in relation to the other components. In this light, identification and characterisation of p53-inducible genes will provide information on the biological function of each gene, as a necessary basis to understand systematic expression data.

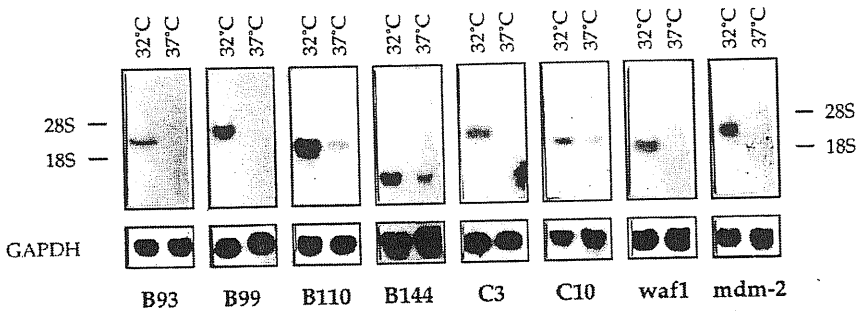


Figure 4
Northern blot analysis of the six regulated clones which were identified in Val5 cells, together with waf1 and mdm2, analysed as controls.

This Thesis describes the characterisation of two novel p53-inducible genes which were originally cloned by a subtractive-hybridisation approach in a murine model system regulated by a temperature-sensitive p53 allele (Val135). The differential screening in Val5 cells, and the preliminary characterisation of six transcripts markedly induced by wt p53 (see Figure 4) have been described by Renè Utrera in his PhD Thesis [238].

Three of the isolated cDNAs displayed significant homology to known genes. Clone B144 corresponded to murine phosphomannomutase (PMM), an enzyme involved in the synthesis of GDP-mannose, required in protein glycosylation. Clone C3, showed some similarity to mitochondrial Carnitine Palmitoyl Transferase-1 (CPT1), an enzyme involved in transport of fatty-acyl CoA across the mitochondrial membrane. Clone B93, encoded a protein similar to N-acetylglucosamine 6-sulphatase (G6S), a lysosomal enzyme involved in the degradation of proteoglycans, mostly heparan-sulphate.

A fourth clone, C10, displayed no homology to any sequence and had no ORF. At the moment it is still uncharacterised.

Clones B99 and B110 are respectively discussed in PART 2 and PART 3 of this Thesis.

PART 2

A NOVEL p53-INDUCIBLE GENE CODING FOR A MICROTUBULE LOCALISED PROTEIN WITH G2-PHASE SPECIFIC EXPRESSION

INTRODUCTION

As already mentioned, many evidences indicate that p53 primarily acts by arresting cells at the G1/S restriction point and this effect has been clearly correlated to induction of Waf1, a potent inhibitor of G1-specific cyclin-dependent kinases [57, 242, 258]. Nevertheless, studies conducted on cells derived from p21Waf1 -/- mice showed that loss of WAF1 only partially abolishes the G1 arrest function associated to wt p53 [20, 48]. So this is likely mediated by multiple pathways, in which other p53-regulated genes might play a role [133]. An important aspect of p53 function regards its possible role in control of the G2 phase. In fact, p53 was shown to interact with the centrosomes [19] and p53-null cells frequently develop an aberrant number of centrosomes [73]. Fibroblasts from p53-/- mice are deficient in the checkpoint that blocks cell-cycle and prevent S-phase entry when cells are treated with spindle inhibitory drugs such as nocodazole [36]. Furthermore, in several cell lines carrying an inducible p53 allele, it has been shown that p53 activation can arrest at both the G1/S and G2/M phases of the cell cycle [3, 229]. While the importance of p21Waf1 in G1 arrest is well established, the role of p21Waf1 in such G2-specific functions is still unclear [48, 128] and it is reasonable to hypothesise that other p53-target genes could be involved. A recent report showed that wtp53 specifically upregulates the sigma member of the 14-3-3 protein family, and that 14-3-3σ is capable of inducing G2/M cell-cycle arrest, possibly by targeting cdc25c phosphatase [75, 104]. It is also worth noting that p53 negatively regulates the expression of MAP4, a microtubule stabilising protein whose intracellular

relocalisation has been correlated to p53-dependent apoptosis [175, 185]. This links p53 to microtubule dynamics and cytoskeletal functions, but also suggests a possible correlation to G2 specific cell cycle events: in fact MAP4 is phosphorylated by cdc2/B kinase at the G2/M transition [186], and progression of the cell cycle through G2/M is linked to the status of microtubules [5].

We screened for p53 target genes in a cellular system in which regulated induction of wt p53 causes an efficient and reversible growth arrest without evidence of apoptosis; in his PhD thesis, Renè Utrera reported the cloning of six cDNAs to be considered as potential p53-responsive genes (1995). I focused my attention on one of these, provisionally named B99, which codes for a novel protein displaying microtubular localisation. I provide evidence that B99 is a direct target for transcriptional activation by p53 and that such induction appears to be restricted to the G2 population of the cells, providing a notable example of a p53 target gene with cell cycle dependent expression. Preliminary analysis in murine cells suggests that B99 is cell-cycle regulated also in the absence of p53 stabilising stimuli, displaying a cyclin-like behaviour. Finally, ectopic expression of clone B99 reduced cell growth and caused a delay at the G2/M phase of the cell-cycle, as determined by flow cytometry. Altogether these evidences indicate B99 as an appealing candidate mediator of specific activities of wtp53 during G2 phase.

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% foetal calf serum (FCS), penicillin (100U/ml) and streptomycin (100µg/ml). Gamma irradiation was performed using a ^{137}Caesium source as described in [47]. UV treatment consisted in a 10J/m^2 irradiation as described in [44]. MMS (Sigma) was added to the culture medium at a final concentration of 25-100µg/ml for 4 hrs, after which the medium was replaced and cells grown for additional 18 hrs before harvesting.

Genomic screening and CAT assays

Approx. 500000 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 hybridised at 40°C in 6XSSC/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 0.2XSSC/0.1%SDS.

For reporter assays, genomic fragments were cloned in the pBLCAT3 vector [146]. Balb/c(10)1 cells plated at low density in 3cm Petri dishes were transfected by the calcium-phosphate precipitation technique with 3µg of promoter constructs plus 0.6µg of p53 expression plasmids (pMSVcL-wtp53 and pMSVKH215-mutant p53) and empty vector to reach a total of 6µg. Cells were collected 24hrs later and analysed for CAT activity by routine procedures [210].

Immunoblotting

Cellular lysates were transferred to 0.2µm pore size nitro-cellulose membrane (Schleicher & Schuell) using a semidry blotting apparatus (BioRad). Detection of B99 protein was performed in high salt conditions (5% dry-milk, 50mM Tris pH=7.5, 1M NaCl, 0,1% Tween-20) for 2 hrs at RT. All other antigens were detected in standard conditions. p21Waf1 was detecetd with a polyclonal rabbit serum raised against a Waf1 synthetic peptide [44] Murine p53 was detected using the PAb240 monoclonal antibody (Oncogene Research). Primary antibodies were detected by incubation for 1hr with horseradish peroxidase (HRPO)-conjugated secondary antibodies (Southern Biotechnology). Blots were developed with the ECL chemioluminescence 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' in cold methanol (-20°C). Alternatively, cells were fixed in PBS/3%PFA for 20' at RT, treated with PBS/1% glycine for 5', and permeabilised in PBS/0.1% Triton-X100 for 5' at RT. 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-b-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 G0/G1 and G2/M fractions (purity >95%) were obtained as described in [47]. Briefly, cells were stained by addition of Hoechst 33342 (10μg/ml) to the culture medium and subsequently sorted on a FACSvantage instrument (Becton-Dickinson) equipped with a 5W argon-ion laser emitting in the UV (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 [43]. Cells were transfected with 7.5μg of pGDSV7-B99 or pGDSV7-Gas2 plasmids by the calcium phosphate precipitation procedure. Approx. 10^5 cells were plated on 6cm Petri dishes and 8 hrs later the DNA precipitate was added. After 12 hrs the precipitate was replaced with DMEM containing 10% FCS, and the cells cultured for additional 48 hrs. 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 [18] for 30' at 37°C. Immune complexes were detected by incubation for 30' with a goat FITC-conjugated anti-rabbit antibody (Sigma) and DNA was stained with 25μg/ml propidium iodide (PI) after RNaseA treatment. Flow cytometric analyses were performed either on a Bryte HS (BioRad) 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 number AJ222580.

RESULTS

p53-dependent regulation of B99 mRNA in murine fibroblasts

In the context of preliminary characterisation, Renè Utrera had 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 ts p53 mutant (Val135) additionally carrying a double point mutation in the transactivation domain that renders it transcriptionally inactive [137] As reported in Figure 1A (column 2), no significant variations in the mRNA levels of B99 occurred in the recipient p53-null cells at 32°C or 37°C (column 1), indicative that upregulation was not a consequence of temperature shift. Importantly, no induction of B99 mRNA was observed in Val135 (25-26) cells shifted to 32°C (column 3), when p53 is in wt conformation, binds to target sites on DNA, but is incapable of transcriptional activity [137]. These data strongly suggested that transactivation by wt p53 is essential for enhanced expression of this clone [238].

I therefore decided to analyse in further detail the transcriptional regulation of B99. 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 stabilisation could be detected upon Cycloheximide treatment of Val5 cells maintained at 37°C (not shown). As can be observed in Figure 1B, B99 mRNA was clearly induced after 6 hrs at 32°C both in the absence or in the presence of 5µg/ml Cycloheximide, as was waf1 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). I 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.

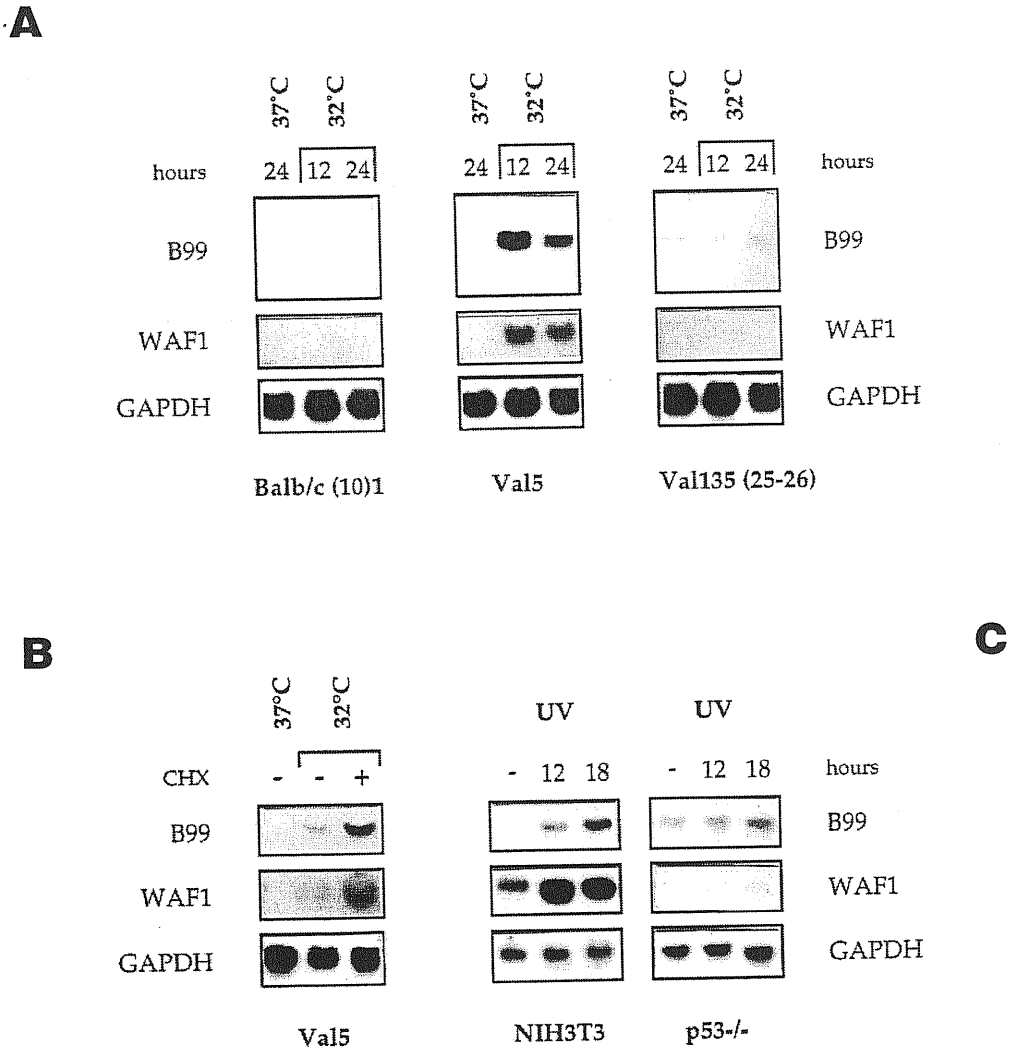


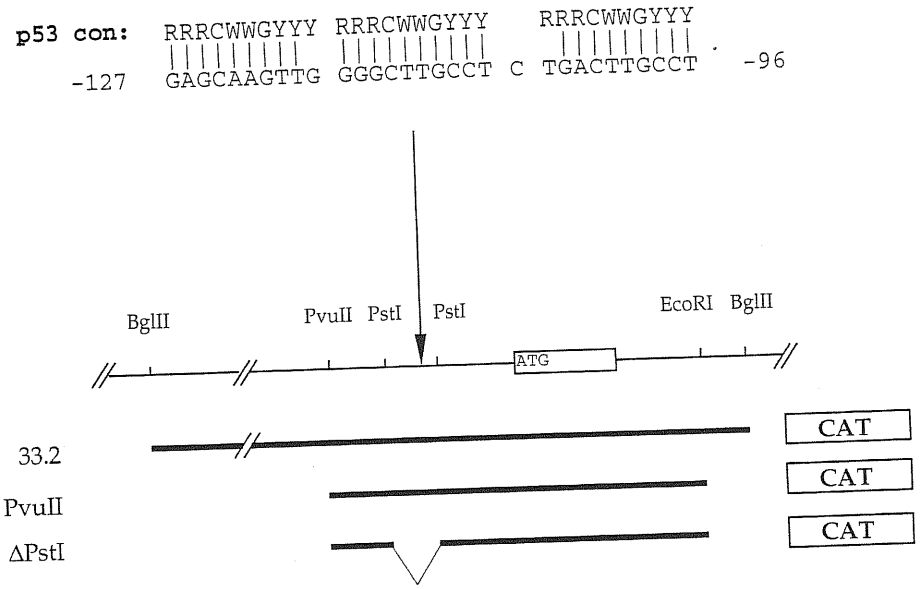
Figure 1
Northern blot analysis of B99 mRNA expression in Val5 and control cell lines.
(A) Total RNA was prepared from the indicated cell lines cultured at 37°C or maintained at 32°C for the indicated time. Balb/c(10)1 are murine fibroblasts with both p53 alleles deleted. Val5 are Balb/c (10)1 cells stably expressing the temperature sensitive 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. Blots were hybridised with a GAPDH probe as a loading control. (B) Regulation in the absence of protein neosynthesis. Total RNA was prepared from Val5 cells grown at 37°C or kept for 6 hrs at 32°C in the absence or in the presence of 5μg/ml of the protein synthesis inhibitor Cycloheximide. (C) Regulation of B99 mRNA in UV treated mouse fibroblasts. Total RNA was prepared from NIH3T3 cells or p53-/- mouse embryo fibroblasts at the indicated time points after UV irradiation (10J/m2). WAF1 mRNA was analysed in the same experiment as a control of p53 activation. Levels of B99 and waf1 are not comparable between the two blots since exposure times were different.

To test whether B99 might be regulated by p53 under more physiological conditions, B99 mRNA expression was analysed in murine fibroblasts subjected to stimuli known to activate endogenous p53 function. For this purpose NIH3T3 cells, which are wild type for p53 [45, 103], and fibroblasts from p53 nullizygous (p53-/-) mice were treated with ultraviolet light (10 J/m^2) and B99 mRNA levels were analysed by Northern blot at 12 and 18 hrs post-irradiation. As shown in Figure 1C, significant induction of B99 mRNA expression was detected in UV irradiated NIH3T3 cells as compared to the basal level in untreated cells. On the contrary, B99 mRNA was not significantly induced by UV treatment in p53-/- MEFs. As a control, waf1 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 NIH3T3, but not in p53-null fibroblasts.

Identification of a p53 responsive element within the B99 gene

To better characterise the p53-dependency of B99 transcriptional regulation, a screening was performed on a mouse genomic library to isolate the B99 promoter. This part of work was done in collaboration with dr. Dejan Lazarevic' and Roberto Verardo, who was an undergraduate student at that time. We selected a lambda clone containing the 5' end of the cDNA. Restriction digests of this clone were Southern blotted and hybridised at low stringency with a labelled degenerated oligonucleotide probe corresponding to the consensus p53 binding sequence described by El-Deiry (see Methods) [56]. A 2.8kb BglII fragment resulted positive to hybridisation; this fragment was isolated and fully sequenced. This genomic segment contained the most 5' sequence of B99 cDNA (its structure is schematically summarised in Figure 2A). An intron was found starting 26bp 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 2A).

A



B

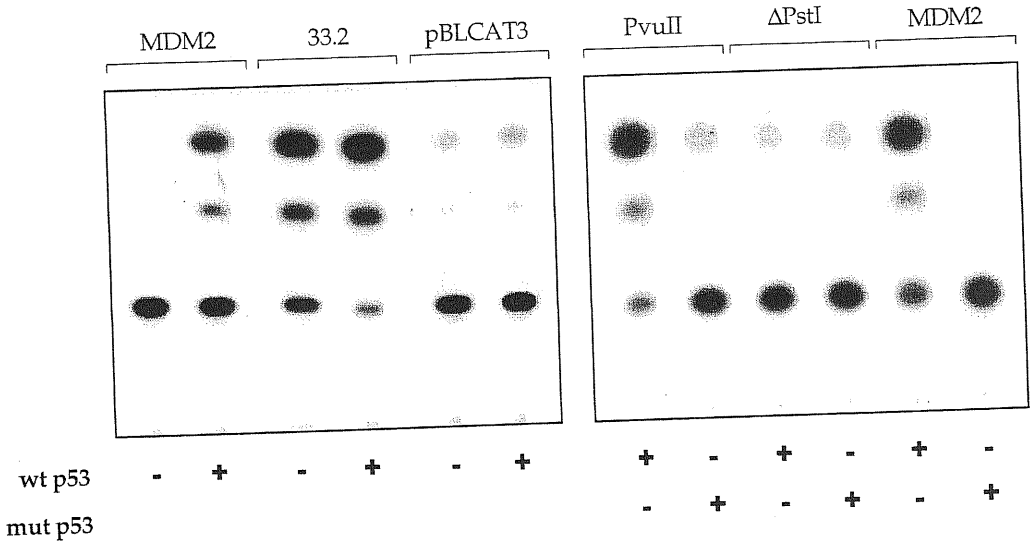
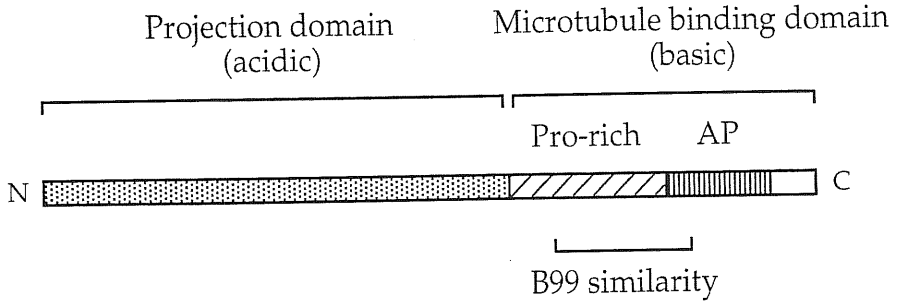


Figure 2
Identification of a p53-responsive element within the B99 gene.
(A) Schematic representation of the genomic clone analysed and structure of the CAT reporter constructs. The position of the identified p53-responsive site is indicated, together with its alignment to the consensus p53-binding sequence (El-Deiry et al., 1992). (B) p53 responsiveness of B99 promoter fragments. The indicated constructs were cotransfected in p53-null Balb/c(10)1 cells with ether wild-type p53, mutant p53, or empty expression plasmids. CAT activity was assayed 24 hrs after transfection. A MDM2 promoter construct (pBP100CAT) was used as a positive control.

The entire BglII 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 wtp53 or the KH215 mutant version [67]. As shown in figure 2B, the genomic segment (33.2) contains a powerful promoter, capable to drive abundant expression of the reporter gene in (10)1 cells. As expected, cotransfection of wtp53 enhanced such expression. Further deletions abolished constitutive transcription from this promoter, unveiling a strong regulation by p53. As shown in figure 2B, a shorter segment (PvuII) starting at nt. -312 with respect to the first ATG was sufficient to confer p53 dependent expression to the CAT reporter. This transactivation was strictly dependent on wtp53 function and was not observed with a mutant p53 protein. When the p53 binding sequence was removed by restriction (ΔPstI) the observed regulation was lost (Figure 2B). We could conclude that a functional p53 responsive element is located in the close vicinity of B99 promoter, providing the molecular basis for the observed regulation by p53.

Characterisation of B99 protein expression in mouse fibroblasts

B99 protein is composed of 741 aminoacids and is rich in positively charged residues. 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 (see Figure 3), a region which has been reported to mediate MAP4 interaction with cyclin B [186]. A polyclonal antibody had previously been raised against a bacterially expressed fragment of B99 protein (see Methods for details); this antibody was employed to determine the intracellular distribution of B99 protein, which appeared to localise to the microtubule network [238].

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          ***  *.* *. . .          * * ** ** ..*  *   *.* . . ..*.   .
MAP4   766 LPSKDVKPKPIADAKAPEKRASPSKPASA-PA-SRS--GSK-STQTVAK-TTTAAAVAST 819
B99    150 LPSSRVQP-PMGEPQLLASPGLLSSPVSAGPAQTQSNQGLPCSSQPLPRESSTSQPPSQA 208

          **  *  * * * .   ..        .*.  * *** . * *  .     ..  *
       820 GPSSRSPSTLLPKKPTAIKTEGKPAEVKKMTAKSVPADLSRPKSTSTSSMKKTTTLSGTA 879
       209 GPQKRITSKLQPPRALPVRGRNLHLATEKL-KKEVPASIQRTKLVNEKG-SQSDVLQDKP 266

            *    ** . *   *    *  . * *  **   *  *.*  ..**     .. *
       880 PAAGVVPSRVKATPMPSRPSTTP-FIDKKPTSAKPSSTTPRLSRLATNTSAPDLKNVRSK 938
       267 STAPDAASR-EGHP-GKRSLPIPGKLGLKKTLLKPPGYTGNLTR-KSSTSG-SASSLESG 322

          *    ..    *  ..  .. .  *. ...  * . .   ** ** *  **   ..  ..
       939 VGSTENIKHQPGGGRAKVEKKTEAAATTRKPESNAVTKTAGPIASAQKQPAGKVQIVSKK 998
       323 V-YRSSV---AGKAKSSEQRSSIPASGSQRRTSTSKSGRIGPAASRQALPAAPARVFGRQ 378
```

Figure 3
Schematic structure of MAP4 and similarity with B99.
MAP4 is divided in a 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). The alignment between B99 and human MAP4 in the region of similarity is also reported.

In order to biochemically characterise the expression of B99 protein in Val5 cells, a kinetic analysis was performed by immunoblotting on extracts prepared 12 and 24 hrs after temperature shift at 32°C. Extracts were also prepared 6, 12 and 24 hrs after shifting the temperature back to 37°C (i.e. p53 to a mutant conformation), condition in which the cells exit p53-induced arrest and promptly re-enter the cell cycle.

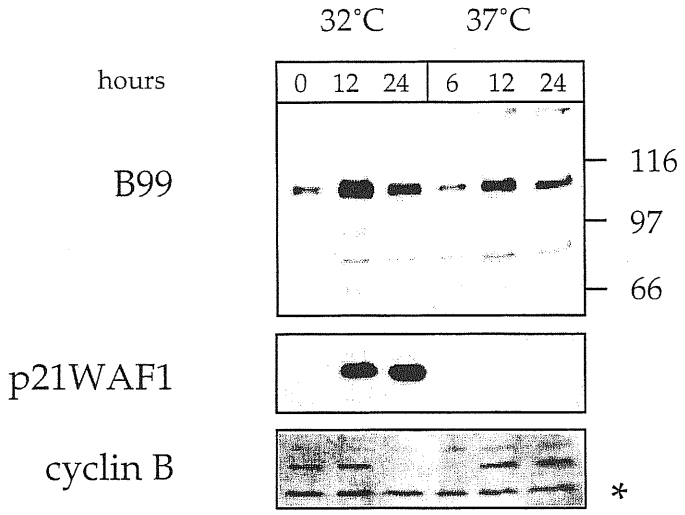


Figure 4
Western blot analysis of B99 protein expression in Val5 cells.
(A) B99 protein regulation in Val5. Cells grown at 37°C for 18 hrs after plating (time 0) were shifted to 32°C for 12 and 24 hrs. Cells arrested by 24 hrs culture at 32°C were then shifted back to 37°C for the indicated times. Cyclin B was analysed as a marker of cell-cycle. The asterisk indicates a cross-reacting protein that serves as internal loading control.

As reported in Figure 4, the antibody detected a specific protein with an apparent molecular weight of 110kDa. B99 protein was strongly induced after 12 hrs at 32°C, while at the 24 hrs time point a noticeable decrease could be observed, in line with the regulation of B99 mRNA (see Figure 1A). A polyclonal antibody to p21Waf1 was used in the same analysis as a control of p53 activation. When Val5 cells were shifted back to 37°C, B99 protein expression was efficiently downregulated within 6 hrs, slightly accumulating again at 12 hrs and returning to basal levels after 24 hrs. 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 G2 phase. As shown in Figure 4, 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 hrs, similarly to what was noticed for B99. This observation suggests that a transient increase in B99 might be associated to passage through the G2 phase during cell-cycle re-entry.

B99 protein expression was also analysed upon DNA damage in mouse cells with or without functional p53. Cells were exposed to UV light, ionising radiation or the alkylating agent methyl methane sulphonate (MMS). Total lysates were prepared 18hrs after treatment and B99 protein levels were analysed by immunoblotting. As reported in figure 5A, B99 clearly accumulated upon DNA damage in NIH3T3, which are wt for p53. Expression of B99 protein was also analysed in NIH3T3 after treatment with PALA, condition in which p53 is activated in response to an unbalance in the cellular pool of ribonucleotides [138]; as shown in figure 5A, B99 was promptly accumulated in treated cells, confirming a tight response to p53 activation.

B99 protein was clearly accumulated upon UV treatment also in C2C12 myoblasts and in C57MG epithelial cells, indicating that it is induced in response to DNA damage in different cell-types (Figure 5B).

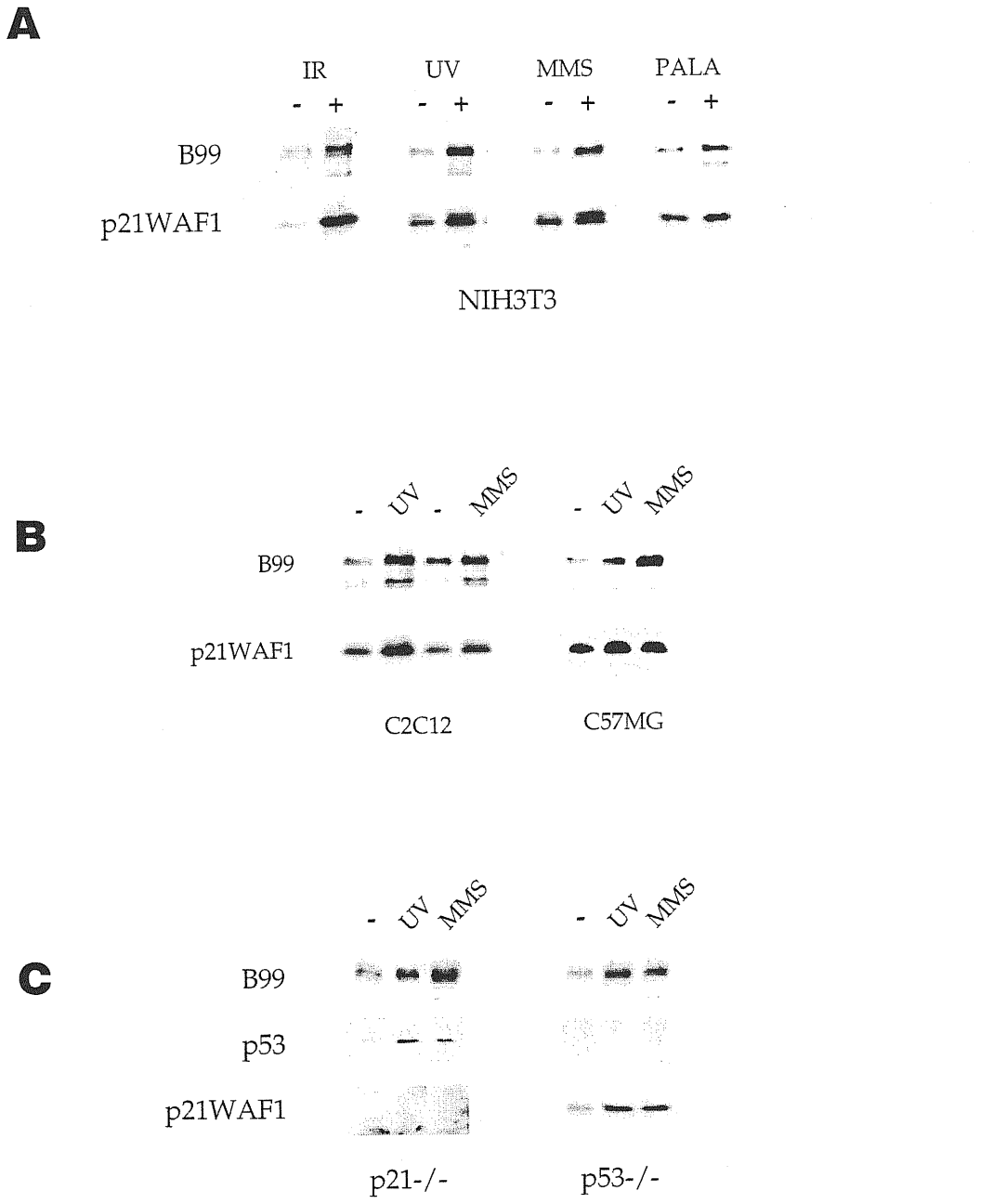


Figure 5
Western blot analysis of B99 protein expression in murine cells.
(A) NIH3T3 fibroblasts were treated with 400rad ionizing radiation (IR), methyl methane sulphonate (MMS) 100µg/ml for 4 hrs, UV-light (10J/m2), or PALA (100µM). (B) C2C12 murine myoblasts and C57MG epithelial cells were treated with UV light or MMS. (C) Primary fibroblasts (MEF) from p53 and p21 knockout mice were exposed to UV-light or MMS. Cells were collected 18 hrs (UV and MMS) or 24 hrs (IR and PALA) after treatment, and total lysates were analysed by immunoblotting with the indicated antibodies.

I also analysed B99 protein expression in primary fibroblasts from p53+/+ and p53-/- mice. As shown in figure 5C, B99 was clearly induced upon UV treatment in cells from p21 nullizygous mice, 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 (see Figure 1C), it is likely that the observed B99 accumulation is due to translational or post-translational regulation. As shown in Figure 5C, the same behaviour was also observed for p21Waf1. These results indicate that similarly to Waf1, B99 can be specifically induced by wtp53 but can also respond to other signalling pathways.

Endogenous B99 is expressed in the G2/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, its expression resulted quite heterogeneous in the cell population analysed. Figure 6A reports two fields of Val5 cells cultured at 37°C or kept for 12 hrs at 32°C and stained with the affinity purified anti B99 antibody: as can be observed, 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 6B. 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 on 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 G2/M subpopulation.

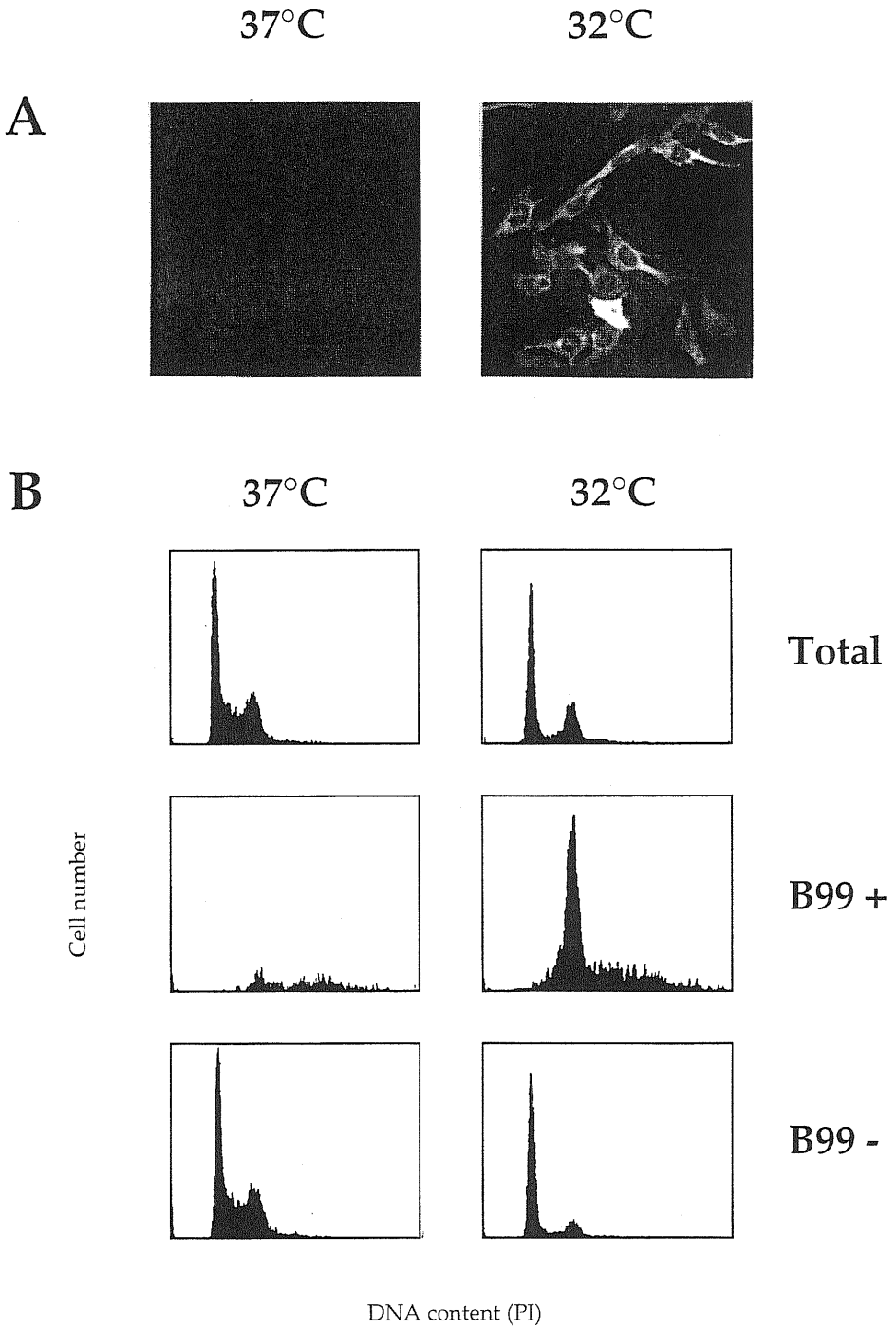


Figure 6
B99 is selectively expressed in the G2/M fraction of arrested Val5 cells.
(A) Expression of endogenous B99 protein in Val5 fibroblasts. Val5 cells growing at 37°C or kept for 12 hrs at 32°C were stained with the affinity purified anti B99 antibody followed by a FITC-conjugated anti-rabbit secondary 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 -).

The same correlation could also be observed at longer periods after temperature shift (i.e. 24 and 48 hrs), 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 6B), indicating that the majority of G2 cells expressed high levels of B99 protein.

To understand if the observed specificity of induction could be extended to a system in which p53 activation was more physiological, I analysed endogenous B99 protein in NIH3T3 mouse fibroblasts after gamma-irradiation treatment. As previously described, irradiation triggers a significant induction of B99 (see Figure 5A). I therefore used a complementary approach to observe the possible correlation between G2 phase and B99 induction already noticed in Val5 cells: the FACS was employed to separate G1 and G2/M cells to >95% purity, as described in [47]. NIH3T3 cells were exposed to 400rad and grown for additional 24 hrs, 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 7B 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 7A, basal levels of B99 protein were detected in the G2/M fraction of untreated NIH3T3 cells. Most notably, upon gamma-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 7A).

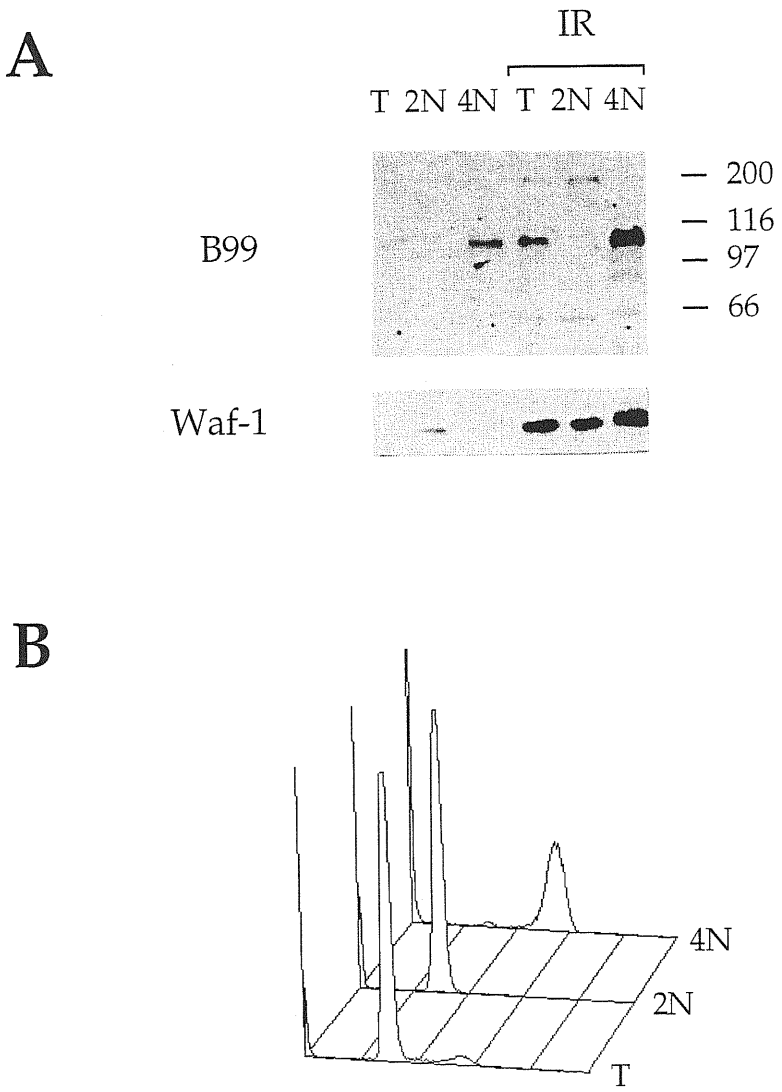


Figure 7
B99 is selectively induced in the G2/M fraction of gamma-irradiated NIH3T3 cells.
(A) Western blot analysis of total (T), G0/G1 (2N), or G2/M (4N) cells separated by FACSvantage sorting on the basis of DNA content before or 24 hrs after IR treatment (400rad). Detection of B99 and p21WAF1 proteins 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.

Cell cycle regulation of B99

The results reported in Figure 7, not only demonstrated that p53-dependent induction of B99 protein is restricted to the cell population with a 4N DNA content, but also suggested that B99 is normally expressed selectively in G2 (see lanes corresponding to untreated cells). To confirm this observation, I decided to analyse B99 expression in NIH3T3 arrested by prolonged culture in 0.5% FCS and stimulated to synchronously re-enter the cell-cycle by addition of 20%FCS. As reported in figure 8A, B99 protein is expressed at undetectable levels in quiescent (G0) cells; its expression is increased at 18 hrs (S phase), and peaks at 22-24 hrs (corresponding to G2-M), to sensibly decrease at longer times, when cells enter G1 of the second cell-cycle, although at this point synchronisation is essentially lost. Comparison with the expression of cyclins A and B suggests that B99 protein has a cyclin-like behaviour, perhaps more similar to cyc A than to cyc B. As shown in figure 8B, the same regulation could be observed for B99 mRNA, suggesting that B99 might be subject to cell-cycle dependent transcriptional control.

To analyse in more detail the specificity of B99 expression, NIH3T3 fibroblasts were synchronised by shaking-off mitotic cells after nocodazole treatment. As shown in figure 9, our antibody detected in mitotic cells a protein with slower mobility than in interphase extracts. This protein is likely to be a modified form of B99, since it was efficiently recognised by the anti-B99 antibody under high stringency conditions (1M NaCl), and could be reproducibly detected in murine extracts enriched for mitotic cells (see also Figures 11 and 12). When metaphase cells are re-plated in the absence of nocodazole, they rapidly complete mitosis and enter G1 phase. As shown in Figure 9, B99 protein was almost undetectable at 2 hrs after replating, confirming that, analogously to S/G2-type cyclins, it is efficiently downregulated in G1 phase.

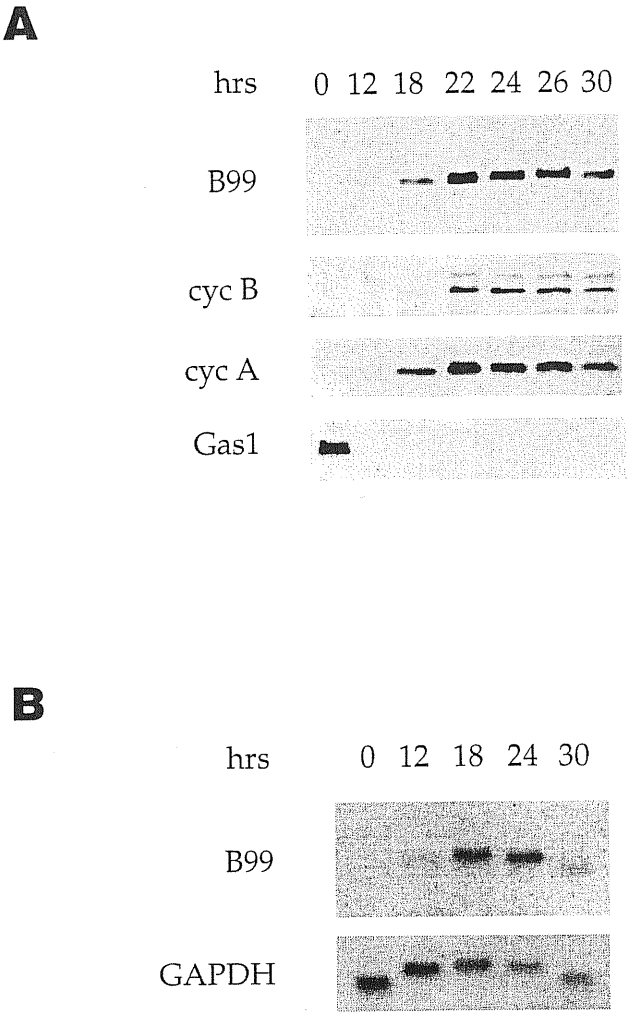


Figure 8
B99 is cell-cycle regulated in NIH3T3 cells.
NIH3T3 fibroblasts were arrested by 48hrs culture in low serum and stimulated to reenter in cycle by addition of 20% FBS. (A) Lysates were collected at the indicated time points and analysed by Western blotting. Equal amounts of total proteins were loaded in each lane. Cyclin B and cyclin A were analysed as markers of cell-cycle progression. The growth-arrest-specific protein Gas1 was analysed as a marker of G0. (B) Total RNA was extracted at the indicated time points and B99 expression was measured by Northern blotting. GAPDH was analysed as a loading control

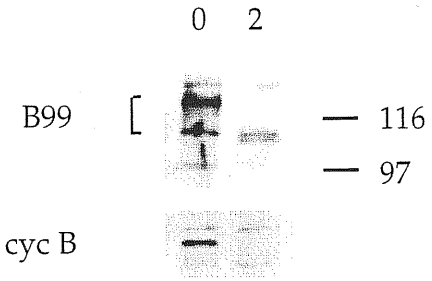


Figure 9
B99 is efficiently downregulated after mitosis. Mitotic NIH3T3 cells were collected by shake-off after Nocodazole treatment (time 0), and analysed 2 hrs after replating in drug-free medium (time 2). Cyclin B was analysed in the same blot as a marker of mitosis. Nocodazole was used at 0.1 µg/ml for 18 hrs. Equal amounts of proteins were loaded in each lane.

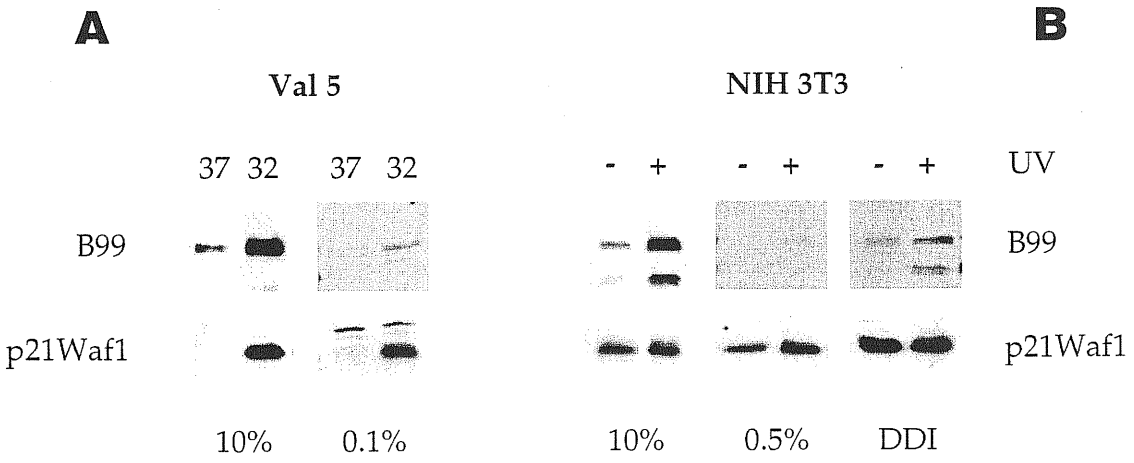


Figure 10
B99 induction in cycling versus arrested cells.
(A) Immonoblot analysis of p53-dependent induction of B99 protein in Val5 cells grown in 10% FBS or arrested by 48hrs culture in 0.1% FBS. For each culture condition, lysates were prepared from cells growing at 37°C or kept 12 hrs at 32°C. (B) Immunoblot analysis of B99 expression upon UV treatment in NIH3T3 cells grown in 10%FBS or arrested either by starvation (48hrs culture in 0.5% FBS) or contact inhibition (DDI). Cells were treated with 10J/m2 and lysates were prepared 18 hrs after irradiation.
p21Waf1 was analysed in the same extracts as a control of p53 activation. Signals are not comparable because of different ECL exposure times.

B99 is not efficiently induced by p53 in quiescent cells

To further investigate the correlation between cell-cycle dependent and p53-dependent regulation of B99 expression, I analysed expression of B99 protein in growth-arrested (G0) cells (Figure 10). Balb/c Val5 fibroblasts were made quiescent by prolonged culture in 0.1% FBS, resulting in a significant decrease of S/G2 cells as assessed by flow-cytometry (not shown). p53 was subsequently activated by shifting to the permissive temperature, and B99 expression was analysed by immunoblotting after 12 hrs. As shown in Figure 10A, induction of B99 protein after conformational activation of wt p53 was extremely less efficient in arrested than in asynchronously growing cells. Waf1 was analysed in the same lysates as a control of p53 activation; although at lower levels, p21Waf1 was induced at 32°C also in arrested cells.

I also analysed B99 induction after DNA damage in NIH3T3 cells made quiescent either by culture in 0.5% FBS for 48 hrs, or by growth to confluence (as in [46]). Dividing and quiescent cells were exposed to UV-light (10J/m2) and lysates were prepared 18 hrs after treatment. As expected, B99 protein was efficiently accumulated in asynchronously growing cells, but induction of B99 was virtually undetectable in serum-starved, and significantly weak in density inhibited cells (Figure 10B). On the contrary, regulation of p21Waf1, analysed as a control, was much less influenced by cell-growth conditions. These data, together with the results in Figure 7, support the notion that B99 is induced by wt p53 only in cells that have traversed the G1/S boundary.

B99 is not induced as a consequence of G2/M cell-cycle arrest

Given the observed G2-specificity, I decided to test whether B99 might be upregulated as a consequence of p53-dependent G2-arrest. To this purpose, asynchronously growing NIH3T3 (bearing wt p53), and Balb/c(10)1 (without p53) cells, were arrested at G2/M by treatment with microtubule-active drugs, and B99 expression was analysed by immunoblotting.

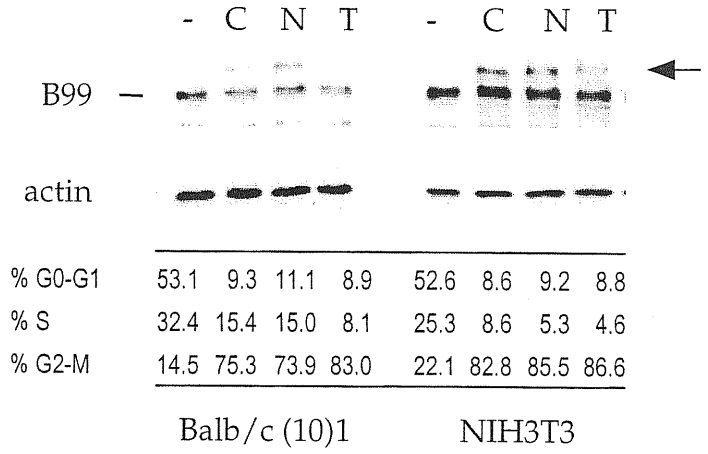


Figure 11
B99 is not upregulated during G2-arrest induced by microtubule-active drugs.
Balb/c(10)1 (p53-null) and NIH3T3 (p53-wt) cells were incubated for 18hrs in the presence of microtubule-active drugs as indicated. Both floating and adherent cells were collected and analysed by flow-cytometry to determine their DNA content. The relative cell-cycle fractions were determined using ModFit (Verity Software House Inc.). Lysates were prepared from the same samples and B99 protein levels were analysed by Western blotting. A monoclonal antibody to a-actin was used as a loading control. C: Colcemid, 0.5μg/ml. N: Nocodazole, 0.125μg/ml. T: Taxol (Paclitaxel), 1μM. The slower migrating band visible in extracts of treated cells (arrow) corresponds to a modified form of B99 specifically observed in mitotic cells (see Figure 10).

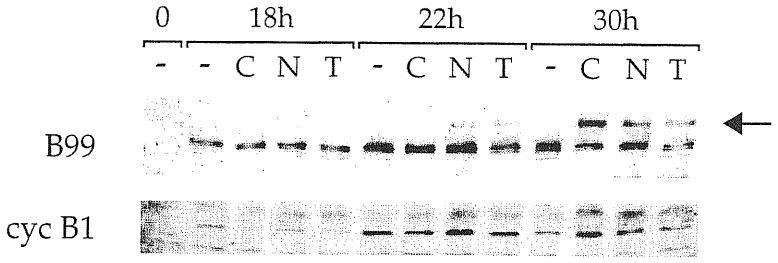
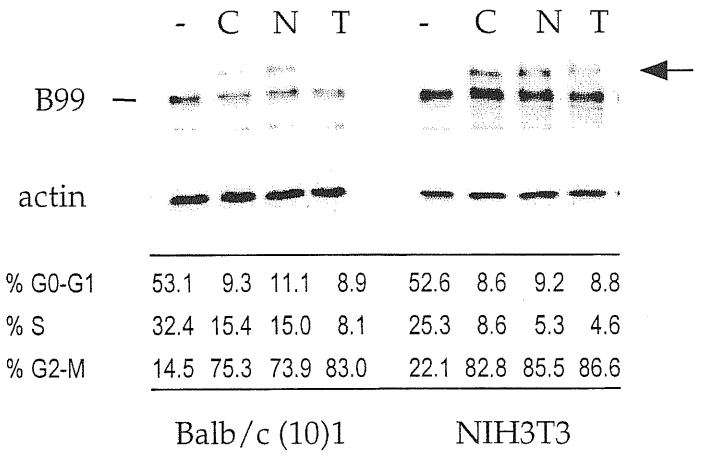


Figure 12
B99 is normally upregulated in the presence of microtubule-active drugs.
NIH3T3 fibroblasts were synchronised by 48 hrs culture in 0.5% FCS (time 0) and ther stimulated to reenter the cell-cycle by addition of 20%FBS in the absence or in the presence of microtubule-poisons. Lysates were prepared at the indicated time points and B99 protein levels was determined by immunoblotting. Cyclin B was analysed in the same blot as a marker of G2-phase. Equal amounts of protein were loaded on each lane. C: Colcemid, 0.5μg/ml. N: Nocodazole, 0.125μg/ml. T: Taxol (Paclitaxel), 1μM. The slower migrating band in extracts of treated cells (arrow) corresponds to a modified form of B99 specifically observed in mitotic cells (see Figure 10).



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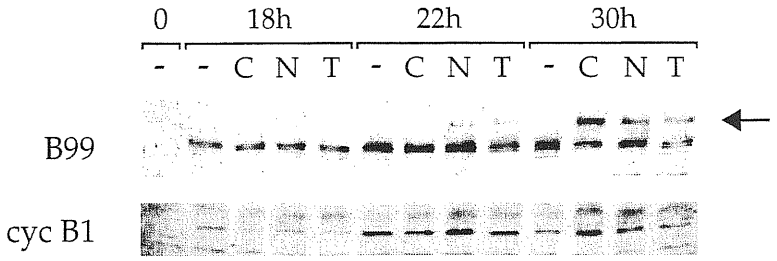

Figure 12
B99 is normally upregulated in the presence of microtubule-active drugs.
NIH3T3 fibroblasts were synchronised by 48 hrs culture in 0.5% FCS (time 0) and ther stimulated to reenter the cell-cycle by addition of 20%FBS in the absence or in the presence of microtubule-poisons. Lysates were prepared at the indicated time points and B99 protein levels was determined by immunoblotting. Cyclin B was analysed in the same blot as a marker of G2-phase. Equal amounts of protein were loaded on each lane. C: Colcemid, 0.5μg/ml. N: Nocodazole, 0.125μg/ml. T: Taxol (Paclitaxel), 1μM. The slower migrating band in extracts of treated cells (arrow) corresponds to a modified form of B99 specifically observed in mitotic cells (see Figure 10).

As shown in Figure 11, under those conditions B99 protein was not upregulated, although approx. 80% of the cells displayed a 4N DNA content. Notably, the mitotic slower migrating form of B99 appeared in such extracts, enriched for metaphase cells (see Figure 9).

Since B99 protein is localised to microtubules, it was possible that drugs affecting tubulin polymerisation could influence B99 regulation. To test this possibility, I analysed B99 expression in NIH3T3 during G0->G2 transition in the presence of microtubule-active drugs (Figure 12). Normal G2-specific induction of B99 protein could be observed in the presence of Nocodazole or Colcemid, while a somewhat lower expression was detected in the presence of Taxol, probably because of higher toxicity of Taxol with respect to the other drugs. I conclude that cell-cycle dependent induction of B99 is not affected by microtubule-active drugs. Therefore, the results of Figure 11 indicate that arresting cells in G2 by blocking transition through M-phase is not sufficient to trigger B99 accumulation.

Cellular localisation of B99 in mitotic cells

Previous studies showed that B99 protein is localised to the microtubule network [238]. Those data were obtained using the anti-B99 antiserum to detect either exogenous B99 overexpressed in p53-null cells, or endogenous B99 protein accumulated in Val5 cells at the permissive temperature. Immunofluorescence analysis of endogenous B99 during cell-cycle in NIH3T3 cells suggested that B99 is localised to the mitotic spindle during metaphase (not shown). To better define this result I prepared a C-terminally tagged version of B99 bearing the vesicular stomatitis virus (VSV) epitope. This construct was transfected in NIH3T3. After transfection, cells were G0-arrested by two days culture in 0.5% FBS, and subsequently stimulated to re-enter the cell-cycle by addition of 20% FBS. Localisation of the exogenous B99 protein was analysed by immunofluorescence with anti-VSV monoclonal antibody at 24-26 hrs after serum addition.

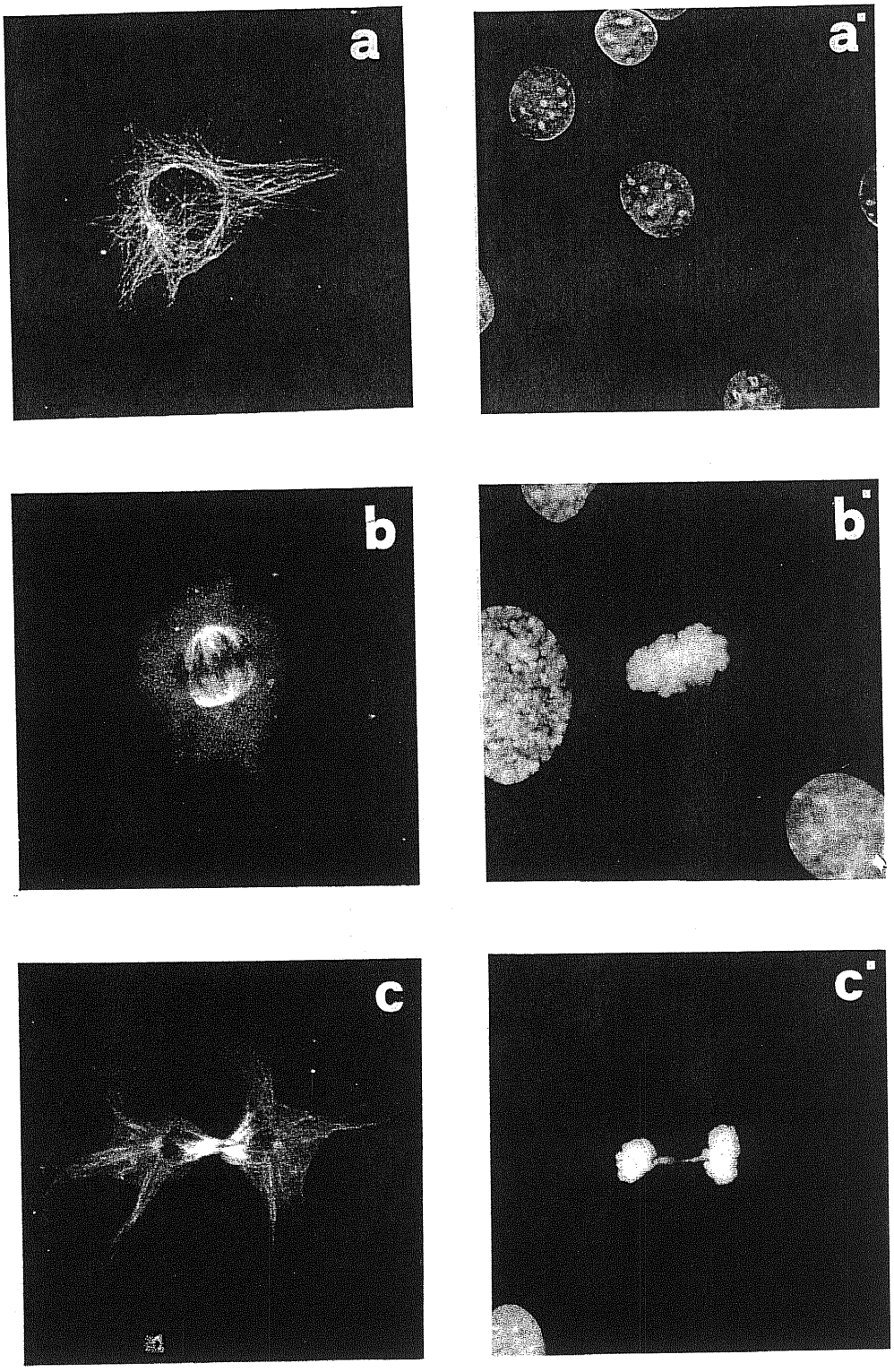

Figure 13
Localization of exogenous B99 in NIH3T3 cells.
Immunofluorescence analysis of transfected C-terminally VSV-tagged B99 at 24 hrs after serum-stimulation of quiescent NIH3T3 cells. (**a**) Interphase, (**b**) Metaphase, (**c**) Telophase cells stained with anti-VSV to detect exogenous B99. (**a'**, **b'**, **c'**) The same microscopic fields, stained with propidium iodide (PI).

VSV-tagged B99 displayed a localisation coincident with the microtubule network in interphase cells (Figure 13A), confirming the previous observations. In metaphase cells, B99 clearly localised to the mitotic spindle, with no particular staining of the astral microtubules (Figure 13B). B99 localised to the spindle also in telophase cells, being mostly detectable in the central interzone region (Figure 13C). Thus, B99 appears to be localised to microtubules not only in interphase, but also during the entire mitotic process.

Ectopically expressed B99 delays G2/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 NIH3T3 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 co-transfected 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 condensed 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 48hrs after transfection, ethanol fixed, and stained for B99. DNA was stained with Propidium Iodide (PI) and the DNA content of B99 expressing cells was determined by application of the appropriate gating on a biparametric cytofluorimetric analysis. The profile of a representative experiment in Balb/c(10)1 mouse fibroblasts is shown in Figure 14, where box B shows the gated, B99 overexpressing sub-population in relation to total cell population reported in box A.

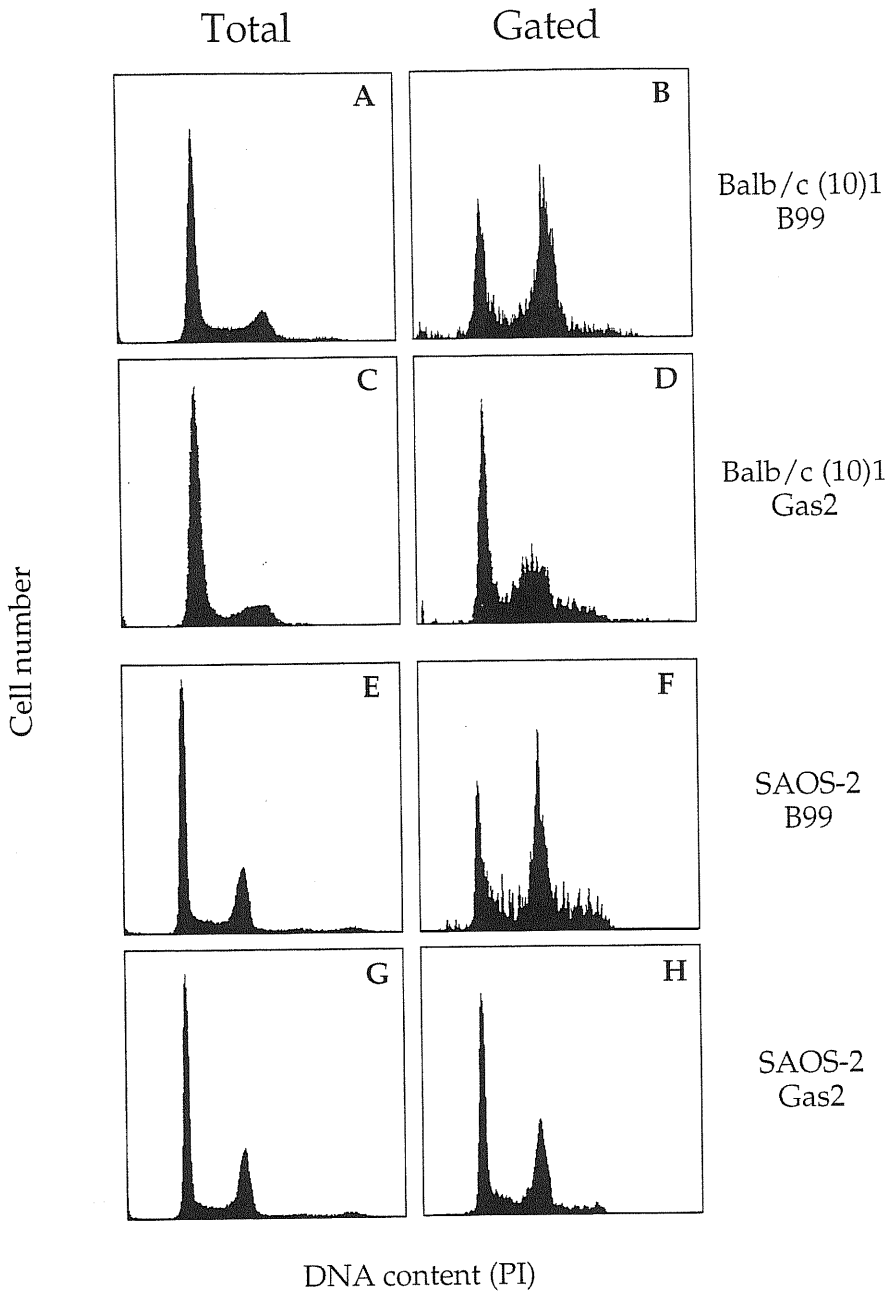

Figure 14
Cell cycle analysis of cells transiently transfected with B99.
Balb/c(10)1 murine fibroblasts and SAOS-2 human osteosarcoma cells were transfected with expression vectors containing B99 or Gas2 under control of the SV40 promoter. Cells were collected 48hrs after transfection, fixed in cold ethanol, incubated with appropriate antibodies and with propidium iodide (to stain cellular DNA) and analysed by flow cytometry. B99 specific staining (FITC) was plotted as a function of DNA content (PI) and an appropriate gating was applied to differentiate the transfected population from the bulk of untransfected cells. A, C, E and G: profile of the total cell populations, obtained by applying no gate. B, D, F and H: DNA content distribution of the gated subpopulations of B99 or Gas2 overexpressing cells.

As can be observed, the cells staining positive for B99 display a larger fraction with a 4N DNA content, indicative of a prolonged G2/M phase. As a control the same cells were transfected with a Gas2 expression vector [18] and as shown in box D, 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 NIH3T3 (not shown) and human SAOS-2 osteosarcoma cells (Figure 14, panels E to H). 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 G1, I confirmed that comparable levels of expression could be obtained in Balb/c (10)1 cells that were arrested at the G1/S boundary by aphidicoline treatment (not shown). Altogether, these data suggest that B99 overexpression delays the G2 phase of the cell cycle.

DISCUSSION

In the present study I have described a cDNA encoding a novel protein, named B99, that is efficiently upregulated in a wtp53 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 wtp53 in the absence of DNA damage. A consensus p53-binding site was identified within the sequences of the B99 gene, and shown to be sufficient to confer wtp53-dependent expression to a reporter gene. Taken together, these data indicate that B99 is a direct transcriptional target of wtp53.

Interestingly, despite B99 mRNA was not upregulated upon UV treatment in p53-null cells, I 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 [141, 149, 165].

When B99 was induced by activation of latent p53 in Val5 cells, it was selectively expressed in the subpopulation with a 4N DNA content. I could also demonstrate that endogenous B99 was specifically induced in the G2/M fraction of NIH3T3 fibroblasts exposed to DNA damage and accordingly, I showed that B99 is poorly induced by p53 in growth-arrested cells. Previous studies suggested that UV-induced p53 accumulation does not occur in G0 cells, although specific DNA binding and SST can still be detected [86, 195]. Nevertheless, I could not detect significant induction of B99 in serum-starved Val5 cells at 32°C, containing high levels of wt p53; this suggests a specific intersection of two separate regulatory pathways, one depending on the cell-cycle, the other on p53 function. To my knowledge, this is the first report of a p53-inducible gene with G2-specific expression, and this selectivity of expression suggests that B99 might be transcriptionally induced 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 [100] and references therein). It has also been shown that p53 can be phosphorylated by different CDKs, and that such phosphorylation modulates its DNA binding activity 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 [235]. The hypothesis that B99 promoter might be regulated by p53 in a cell-cycle dependent fashion is an interesting issue that will be subject of future work.

Through cell-cycle analysis I could demonstrate that B99 is maximally expressed at late S/G2 phase in NIH3T3 cells, and is downregulated in G1, similarly to cyclin A or

B. It is worth noting that B99 was not accumulated in Balb/c(10)1 or NIH3T3 cells arrested at G2/M by microtubule-active drugs such as Nocodazole or Taxol, thus indicating that B99 upregulation is not a consequence of G2/M arrest, but actually requires specific signals.

Finally, when B99 was transfected in p53-null murine fibroblasts it showed a growth suppressive effect and interfered with G2/M phase progression, as documented by the increased fraction with 4N DNA content in cells overexpressing exogenous B99.

Very poor hints on the possible biochemical function of B99 protein could be drawn from its primary sequence and intracellular localisation. B99 protein is 741 aminoacids long and is rather basic. It was shown to localise to the microtubule network but lacks obvious microtubule interaction motifs. Further biochemical characterisation 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 (see 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 [186]. Interestingly, wtp53 has been reported to specifically repress MAP4 transcription in Val5 fibroblasts, and p53-mediated downregulation of MAP4 has been functionally correlated to p53-dependent apoptosis [28]. Accordingly, MAP4 overexpression was shown to interfere with p53-induced apoptosis, but was reported to have no effect on cell cycle arrest [28]. Thus, the microtubule-localised B99 protein is induced by wtp53 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, increasing evidences accumulated indicating an involvement of p53 in the control of cell-cycle events that occur past the G1/S transition. Indeed, a complex pattern of still poorly characterised wtp53-dependent functions is being gradually uncovered through different experimental approaches. Loss of p53 function has been clearly

correlated to abnormal centrosome amplification [19, 73]. Most importantly, it has been shown that p53 can activate a G2/M cell cycle block [93, 96], and that wtp53 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 [36, 128]. It has been reported that such p53-dependent spindle checkpoint requires p21Waf1 induction [128], but previous work suggested that it was still functional in fibroblasts from p21 nullizygous mice [3]. It has been proposed that p53-induced G2 arrest could be mediated through accumulation of p21Waf1, which binds the mitotic cdc2/B kinase and could be inhibiting MPF activity [3, 135, 258]. Moreover, it has also been proposed that G2/M cell cycle arrest could result from p53-dependent induction of 14-3-3s protein, a negative regulator of cdc25c [75, 103].

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 G2/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 (see Figure 6). As previously shown for other cellular systems, it is likely that p53 is arresting or delaying these cells in the G2/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 G2/M progression. On the basis of the above observations I propose B99 as a candidate mediator of G2-specific functions of wtp53.

PART 3

WT p53 DEPENDENT EXPRESSION OF A MEMBRANE-ASSOCIATED ISOFORM OF ADENYLATE KINASE

INTRODUCTION

As described in PART 1, wild-type p53 regulates transcription of a number of cellular genes. One important example is Waf1, largely responsible for the G1-arrest function of wt p53 [48, 57, 258]; while other p53-induced genes are directly or indirectly involved in apoptosis, angiogenesis, and G2/M cell-cycle arrest. Nevertheless, many genes that appear to be p53-inducible cannot be easily correlated to cell-cycle regulation or apoptosis, and it is legitimate to hypothesise that complex patterns of multiple p53-regulated genes, acting on different pathways, co-operate to mediate a final biological effect.

As mentioned, we performed a subtractive hybridisation screening in Val5 cells, and therein isolated six p53-inducible transcripts [238]. At the permissive temperature, Val5 cells undergo a p53-dependent G1 arrest which can be maintained for several days in culture, and is readily reversible when cells are returned to the non-permissive temperature [44]. Thus, according to the above hypothesis, the particular combination of genes induced by wtp53 in Val5, is instrumental in attaining a "reversible and prolonged cell-cycle arrest" phenotype. I report here that one of those transcripts encoded a previously undescribed form of Adenylate Kinase, displaying plasmamembrane localisation.

Adenylate kinase (Myokinase, EC 2.7.4.3) is a ubiquitous monomeric enzyme which catalyses the reversible transfer of phosphate between adenine nucleotides, according to the reaction ATP+AMP=ADP+ADP [178], and is fundamental to regulate

homeostasis of adenine nucleotides in the cell. In vertebrates three isozymes have been characterised: AK1 is cytoplasmic; AK2 and AK3 are localised in the intermembrane space of mitochondria ([232] and references therein). The alternative protein I describe here is identical to cytosolic AK1, with only the addition of a N-terminal stretch of aminoacids mediating membrane localisation, possibly by lipid modification.

The enzymology and molecular structure of cytoplasmic AK1 are extremely well known (see [24, 53]), but very little is known about regulation of AK1 expression in fibroblasts, or about possible functions of AK1 in modulating cell-cycle events. I describe antisense experiments in Val5 suggesting that induction of the alternative AK1β protein plays a critical role in the kinetic performance of p53-dependent cell-cycle arrest.

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% foetal calf serum (FCS), penicillin (100U/ml) and streptomycin (100µg/ml). UV treatment consisted in a 10J/m^2 irradiation as described in [44].

Northern blotting

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 [27]. The oligonucleotide probe used to detect the alternative AK1β mRNA in Northern blots had the following sequence: 5'-GGTTCACTAGACACACA GCAGCCCATGATCCTA-3'.

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).

Production of polyclonal antisera, Immunoblotting and Immunoprecipitation

Purified porcine AK1 was purchased from Sigma. Rabbits were injected with 200 µg of denatured AK1 cut out from a 15% acrylamide/SDS electrophoresis gel, mixed 1:1 with complete Freund's adjuvant (Sigma). The same amount of protein was subsequently injected every 21 days for 4 months. The synthetic peptide (N-CVSSEPQEEGGRKTGEKL-C) corresponding to the alternative N-terminal sequence of AK1β was conjugated to Keyhole Limpet Haemocyanin (Sigma) and 200µg of peptide-KLH mixed 1:1 with Freund's adjuvant were injected every 21 days for approximately 6 months.

Immunoblots were routinely performed at RT in Blotto-Tween under standard conditions. Fyn was detecetd using a rabbit polyclonal anti-Fyn antibody (Santa Cruz). Tubulin was detecetd using a monoclonal antibody anti-a tubulin (Sigma). Primary antibodies were revealed with horseradish peroxidase (HRPO)-conjugated secondary antibodies (Southern Biotechnology). Blots were developed with the ECL chemioluminescence system (Amersham).

For immunoprecipitation, Val5 cells were labelled with 35S-Methionine for 4 hrs. Cells were scraped in denaturing buffer (0.8% SDS, 150mM NaCl, 50mM Tris pH=7.5) and boiled for 5 min. SDS was subsequently quenched by adding a volume of quenching buffer (4% TritonX100, 150mM NaCl, 50mM Tris pH=7.5) containing protease inhibitors. After preclearing, the crude anti-AK1 serum was added and incubated overnight at 4°C. Immune complexes were then collected on Protein A-sepharose beads (Pharmacia), separated by SDS/PAGE, and exposed to autoradiography.

Preparation of membrane and cytosolic fractions

Membranes and cytosol fractions were prepared as described [115], with minor modifications. Subconfluent Val5 cells were scraped in ice-cold phosphate-buffered saline (PBS) and pelleted by centrifugation. Cells were resuspended in 1 ml of 6% (w/w) sucrose -10mM Pipes, pH 7.2 plus proteinase inhibitors, and disrupted by sonication. Sonicated cell extracts were centrifuged at 600 g for 3 min to remove nuclei and unbroken cells. Supernatants were loaded on sucrose gradients prepared by layering 2 ml of 47, 40, 30 and 20% sucrose on a 55% cushion and centrifuged at 200000 g for 2 hours. The top cytosol fraction and the 20-30% interphase were collected. The latter was pelleted at 100000 g for 30 min and resuspended in 9% sucrose containing proteinase inhibitors. Protein concentrations were measured by the Bradford assay (BioRad) and equal amounts of extracts from the two growth-conditions were loaded for each fraction.

Immunofluorescence Microscopy

Cells were plated on glass coverslips within 3 cm tissue culture dishes. After washing with PBS, cells were fixed in PBS/3%PFA for 20' at RT, treated with PBS/1% glycine for 5', and permeabilised in PBS/0.1% Triton-X100 for 5' at RT. AK1 proteins were detected with a monoclonal anti-VSV antibody (Sigma) followed by a goat anti-mouse FITC-conjugated antibody (Sigma). Human TR was detecetd using the OKT-9 monoclonal antibody. For S-phase quantitation, cells were incubated with 50μM BrdU prior to fixation, and a short treatment with 50mM NaOH was performed to denature DNA before incubation with primary antibodies. BrdU was detected using a monoclonal IgG2a antibody (Amersham). After incubations and extensive washes, coverslips were mounted on glass slides and analysed using a laser scan confocal microscope (Zeiss).

RT-PCR analysis of human AK1 trancripts

Random primed cDNAs were produced from 10μg of total RNA. Dilutions of the cDNA were prepared and identical aliquots were used for PCR amplification with two separate set of primers. The alternative 5' end of human AK1β was amplified using the primers UP: 5'-GGAATTCGACCATGGGCTGCTGCTC-3', and LO: 5'-GGAATTCGCAG CAGTGTGGGCTGTC-3'. An internal fragment of human GAPDH was amplified using the primers UP: 5'-CATGCCATCACTGCCACCC-3', and LO: 5'-ACCTGGTCCTCAGTGTAGC-3'. Amplification products were separated on agarose gels and analysed by Southern blotting using either a probe corresponding to the ORF of murine AK1 (cross-reactive to human AK1β) or a probe corresponding to rat GAPDH.

Accession numbers

The cDNA sequences of murine AK1 and AK1β have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AJ010108 and AJ010109.

RESULTS

Isolation of murine AK1 by subtractive hybridisation

Val5 is a cell line derived from p53-deficient Balb/c (10)1 mouse fibroblasts [96] by stable transfection of the temperature sensitive Val135 allele of murine p53 [157, 164] At the permissive temperature of 32°C these cells upregulate p53 transcriptional targets such as waf1 or mdm-2, and undergo an efficient and reversible G1 arrest [44]. During a differential screening designed to detect novel p53-regulated genes, six cDNAs were isolated that are markedly induced by wtp53 in the Val5 cell line. One of the isolated clones, 1.1 Kb in length and provisionally named B110, corresponded to a transcript that was estimated to be approximately 2Kb long according to Northern blot analysis. No ORF was present, and database homology search did not reveal any similarity to known sequences. By further screening of a 32°C-specific Val5 cDNA library using the B110 probe, two different nearly full-length clones were obtained. One of them, named B110.N6, corresponded to the murine homologue of cytosolic Adenylate Kinase, AK1 (EC 2.7.4.3). Indeed, the corresponding protein is encoded in a short open reading frame not contained in the original B110 clone (Figure 2A).

Induction of B110 mRNA is dependent upon p53 transcriptional function

Expression of B110 was analysed by Northern blot, comparing its regulation in Val5 cells (37°C vs. 32°C) with its expression in the recipient Balb/c (10)1 and in Balb/c Val135(25-26) cells. The Val135(25-26) is a cell line derived from p53-null Balb/c (10)1 fibroblasts by stable transfection of a ts p53 mutant (Val135) carrying two additional point mutations in the transactivation domain that render it transcriptionally inactive [137]. As shown in Figure 1A, B110 mRNA was strongly induced in Val5 cells cultured at 32°C for 12 or 24 hrs.

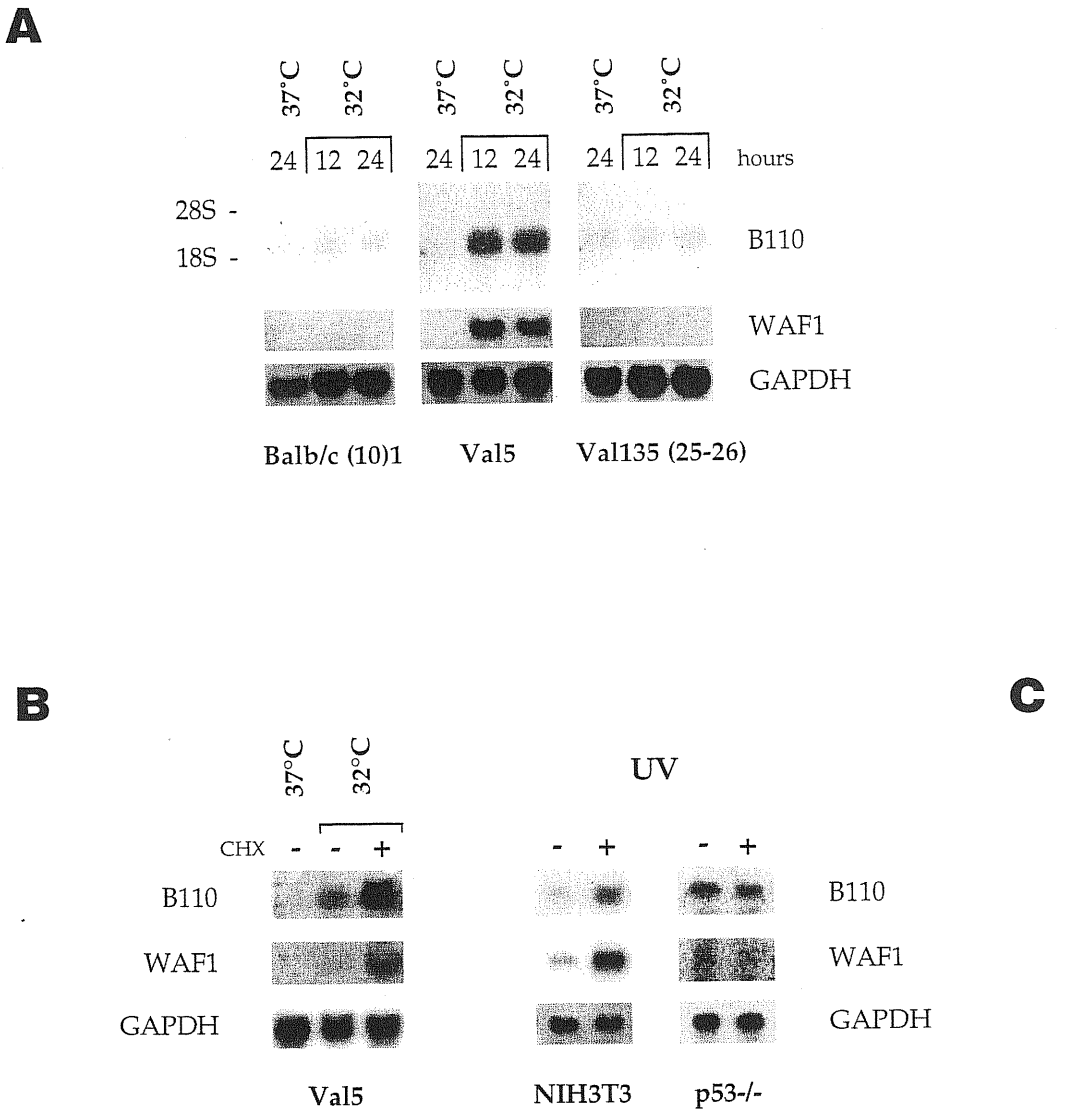

Figure 1
Northern blot analysis of AK1 mRNA expression in Val5 and control cell lines.
10 µg of total RNA were loaded on each lane and hybridisation was performed under high stringency conditions. (A) Regulation in control cells. RNA was prepared from the indicated cell lines cultured at 37°C or mantained 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 temperature sensitive 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 hrs at 32°C in the absence or in the presence of 5µg/ml of the protein synthesis inhibitor cycloheximide. (C) Regulation of AK1 mRNA in UV treated mouse fibroblasts. Total RNA was prepared from NIH3T3 cells or p53-/- mouse embryo fibroblasts (MEF) 18 hrs after UV irradiation. WAF1 mRNA was analysed as a control of p53 activation. Levels of AK1 and waf1 are not comparable between the blots since exposure times were different.

No significant variations in B110 mRNA levels were observed in the recipient Balb/c (10)1 cells at 32°C, indicating that upregulation was not a consequence of temperature shift. Importantly, no induction of B110 mRNA was observed in Val135(25-26) cells at 32°C, when p53 is in wt conformation, binds to target sites on DNA, but is incapable of transcriptional activity [137]. This suggests that transactivation by wt p53 is required for enhanced expression of B110 mRNA. As a control, the same Northern blots were hybridised with a murine waf1 probe (Figure 1A).

To test whether the observed induction might be a secondary consequence of p53 activation, expression of B110 mRNA was analysed in Val5 cells in the absence of *de novo* protein synthesis. As shown in Figure 1B, B110 mRNA was clearly induced after 6 hrs at 32°C even in the presence of 5μg/ml Cycloheximide, as was waf1 mRNA analysed as a control. Under these conditions protein synthesis was efficiently inhibited, as determined by lack of p21Waf1 protein induction (not shown). Thus, p53-dependent accumulation of B110 mRNA occurs in the absence of protein neosynthesis, providing indirect evidence that B110 is a transcriptional target of wtp53 in these cells.

To test whether B110 might be regulated by p53 under more physiological conditions, mRNA expression was analysed after stimuli known to activate endogenous p53 function in mouse fibroblasts. For this purpose NIH3T3 cells, which are wild type for p53 [45], and fibroblasts from p53-nullizygous mice were treated with ultraviolet light (10 J/m^2) and B110 mRNA levels were analysed by Northern blot 18 hrs after irradiation. As shown in Figure 1C, significant accumulation of B110 mRNA was detected in NIH3T3 while no induction could be observed in p53-null fibroblasts. A probe to waf1 was used as a control of p53 activation. Thus, UV induced DNA damage caused upregulation of B110 mRNA in a wtp53-dependent manner in murine cells.

A

```
murine AK1   MEEKLKKAKIIFVVGGPGSGKGTQCEKIVQKYGYTHLSTGDLLRAEVSSG 50
             ||||||| |||||||||||||||||||||||||||||||.|||||
human AK1    MEEKLKKTKIIFVVGGPGSGKGTQCEKIVQKYGYTHLSTGDLLRSEVSSG

             SERGKKLSAIMEKGELVPLDTVLDMLRDAMLAKVDSSNGFLIDGYPREVK 100
             | |||||| |||||:||||:||||||||||.|||..| ||||||||||.
             SARGKKLSEIMEKGQLVPLETVLDMLRDAMVAKVNTSKGFLIDGYPREVQ

             QGEEFEQKIGQPTLLLYVDAGAETMTQRLLKRGETSGRVDDNEETIKKRL 150
             |||||||.:|||||||||||||| |||||||||||||||||||||||||
             QGEEFERRIGQPTLLLYVDAGPETMTQRLLKRGETSGRVDDNEETIKKRL

             ETYYNATEPVISFYDKRGIVRKVNAEGTVDTVFSEVCTYLDSLK       194
             |||| ||||||.||:||||||||||||.||.|||:|||:||.||
             ETYYKATEPVIAFYEKRGIVRKVNAEGSVDSVFSQVCTHLDALK
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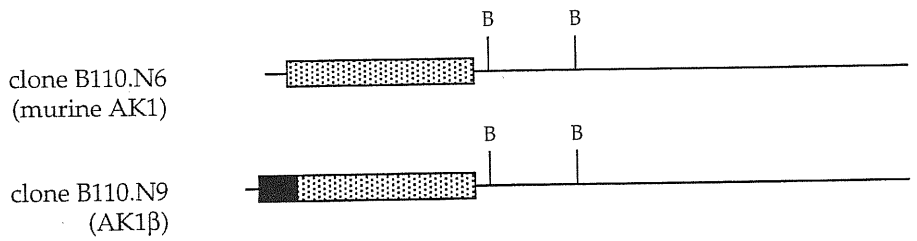
B

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AK1                          ME EKLKKAKIIFVVGGPGSGKGTQ...
AK1β       MGCCVSSEPQEEGGRKTG EKLKKAKIIFVVGGPGSGKGTQ...
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Figure 2
Two different cDNA clones corresponding to the B110 probe.
(A) Clone B110.N6 encodes murine cytosolic Adenylate kinase. Alignment of the deduced peptide encoded by clone B110.N6 with the human AK1 protein. (B) Clone B110.N9 encodes an alternative form of AK1. The two clones are schematically drawn to show the identical 3'UTRs and the alternative 5' sequence in clone B110.N9 (black box). The aligned N-terminal aminoacidic sequences of both deduced proteins are also shown. (B = BamHI).

wt p53 induces expression of an alternative AK1 mRNA

As already mentioned, a second cDNA clone was isolated during the screening of a Val5 cDNA library with the B110 probe. Such clone, named B110.N9, was determined by restriction analysis and partial sequencing to be almost identical to clone B110.N6, differing only in approximately 90 nucleotides in the 5' region. The structure of the two clones is schematically shown in Figure 2B. Conceptual translation of clone B110.N9 resulted in a deduced peptide starting from a different ATG on the same reading frame, and in which 18 extra aminoacids are added to the N-terminus of the AK1 protein (see Figure 2B). This alternative product was named AK1β.

To test if the identified mRNA was actually expressed in Val5 cells at the permissive temperature, I designed an antisense oligonucleotide on the 5' sequence of clone N9 and used it as a probe for Northern blot analysis. As shown in figure 3A, the N9-specific oligonucleotide probe recognised a regulated transcript, selectively expressed at 32°C. This result confirmed that the alternative form corresponding to AK1β was indeed expressed, and was efficiently regulated by p53.

wtp53 induces expression of an alternative AK1 protein

To study AK1 expression at the protein level, I produced a rabbit polyclonal antiserum against porcine AK1. Expression of AK1 protein was analysed by immunoblotting on extracts from Val5 cells grown at 37 or 32°C; as can be observed in Figure 3B, cytoplasmic AK1 did not appear to be significantly regulated. Rather, a slower migrating band detected by the same antibody was clearly accumulated at 32°C, albeit with a significantly slower kinetics than p21Waf1, analysed as a control. To better evaluate the regulation of both AK proteins, I decided to immunoprecipitate Adenylate Kinase from Val5 cells grown at 37°C or at 32°C after pulse labelling with ^{35}S methionine. As can be seen in figure 3C, cytoplasmic AK1 was not strongly regulated, displaying a similar rate of synthesis at both temperatures. On the contrary, the slower migrating band was only detected at 32°C, indicating specific induction.

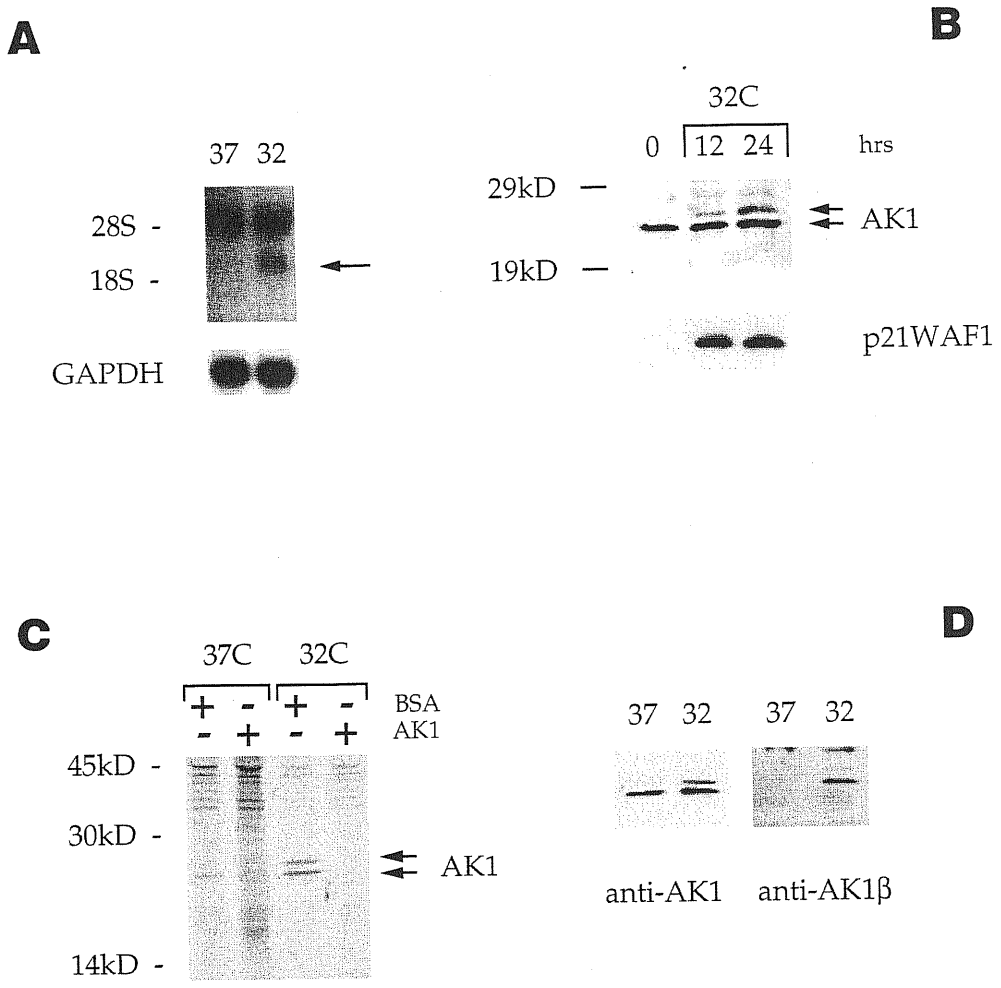


Figure 3
The alternative AK1b protein is induced by wt p53 in Val5.
(A) Northern blot analysis of total RNA from Val5 cells at 37°C or after 12 hrs at 32°C. AK1b mRNA was detecetd using a labelled oligonucleotide specific for the alternative 5' sequence. (B) Western blot analysis of AK1 expression in Val5 fibroblasts. Subconfluent cells growing at 37°C (time 0) were shifted to 32°C for 12 and 24 hrs. Equal amounts of extracts were analysed by immunblotting with the anti-AK1 polyclonal antiserum. p21Waf 1 was analysed in the same blot as a marker of p53 activation. (C) Immunoprecipitation analysis of metabolically labelled AK proteins in Val5 cells. AK proteins were immunoprecipitated with anti-AK1 serum under denaturing conditions from Val5 cells grown at 37°C or kept for 12 hrs at 32°C, after labelling with 35S methionine for 4 hrs before harvesting. The serum was pre-incubated with 200μg/ml of either BSA or porcine AK1 as indicated. (D) Western blot analysis of AK1β expression in Val5 cells. Lysates from Val5 grown at 37°C or kept for 24 hrs at 32°C were analysed by immunoblotting using either the anti-AK1 antiserum, which detects both AK proteins, or the anti-AK1β affinity purified antibody, which only detects the alternative AK1β protein.

This band migrated with the same electrophoretic mobility of *in vitro* translated AK1β (not shown). This same band was specifically recognised by the anti-AK1 antibody, since it could not be detected when the serum was pre-incubated with purified AK1 (Figure 3C). Therefore, upon activation of p53 function in Val5 cells, a slower migrating form of AK1 was specifically induced, possibly corresponding to the alternative protein encoded by clone B110.N9.

I therefore decided to produce a polyclonal antiserum against a synthetic peptide corresponding to the first 15 aminoacids of AK1β. This antibody was affinity purified on the respective peptide, and used for immunoblotting on extracts of Val5 cells. As shown in Figure 3D, the AK1β-specific antiserum detected a protein selectively expressed at 32°C in Val5. Taken together, these results strongly imply that the slower migrating band recognised by the anti-AK1 serum is indeed AK1β.

The alternative AK1β protein is localised to the plasmamembrane

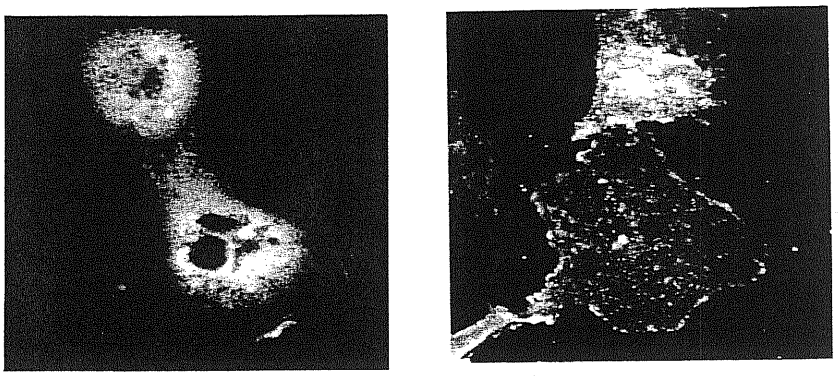
Analysis of the N-terminal extra aminoacids of AK1β suggested the presence of a putative signal for N-myristoylation; in fact, as shown in Figure 4A, the N-terminus of AK1β contains the conserved residues involved in myristoylation (and palmitoylation) of *src* -like protein kinases and α subunits of heterotrimeric G-proteins (see [201] and references therein). Cotranslational addition of myristate at the N-terminus of these proteins has been described to be instrumental for their localisation to the membrane compartment; therefore, I addressed the possibility that AK1β could be a membrane-associated form of Adenylate Kinase.

To compare the intracellular localisation of AK1 and AK1β, the two proteins were tagged with a C-terminal VSV epitope. These constructs were cloned in a mammalian expression vector and transfected in COS-7 cells. Expressed proteins were stained with a monoclonal antibody to the VSV epitope and analysed by confocal microscopy. As shown in figure 4B, the AK1 protein appeared diffused in the cytoplasm. On the contrary, the longer AK1β protein displayed a clear membrane-bound localisation.

A

src	M	G	S	S	K	S	K	P	K	D	P	S	Q	R	R	R
lyn	M	G	C	I	K	S	K	R	K	D	N	L	N	D	D	E
yes	M	G	C	I	K	S	K	E	D	K	G	P	A	M	K	Y
fyn	M	G	C	V	Q	C	K	D	K	E	A	T	K	L	T	E
yrk	M	G	C	V	H	C	K	E	K	I	S	G	K	G	Q	G
hck	M	G	C	M	K	S	K	F	L	Q	V	G	G	N	T	F
fgr	M	G	C	V	F	C	K	K	L	E	P	V	A	T	A	K
lck	M	G	C	V	C	S	S	N	P	E	D	D	W	M	E	N
blk	M	G	L	L	S	S	K	R	Q	V	S	E	K	G	K	G
Gαi1	M	G	C	T	L	S	A	E	D	K	A	A	V	E	R	S
Gαo	M	G	C	T	L	S	A	E	E	R	A	A	L	E	R	S
Gαz	M	G	C	R	Q	S	S	E	E	K	E	A	A	R	R	S
AK1β	M	G	C	C	V	S	S	E	P	Q	E	E	G	G	R	K

B



AK1 -VSV AK1β -VSV

Figure 4
The alternative AK1β protein is localised to the plasmamembrane.
(A) Alignment of the N-terminus of AK1β with several myristoylated proteins. The upper block includes members of the src-like family of protein kinases. Le lower block includes the Ga subunits of trimeric G-proteins. The highly conserved residues are boxed. (B) Cellular localisation of ectopically expressed AK proteins. COS-7 cells were transfected with plasmids expressing VSV tagged AK1 (B) or AK1β (C) proteins. 48 hrs later cells were fixed, stained with a monoclonal anti-VSV antibody, and photographed using a confocal microsope

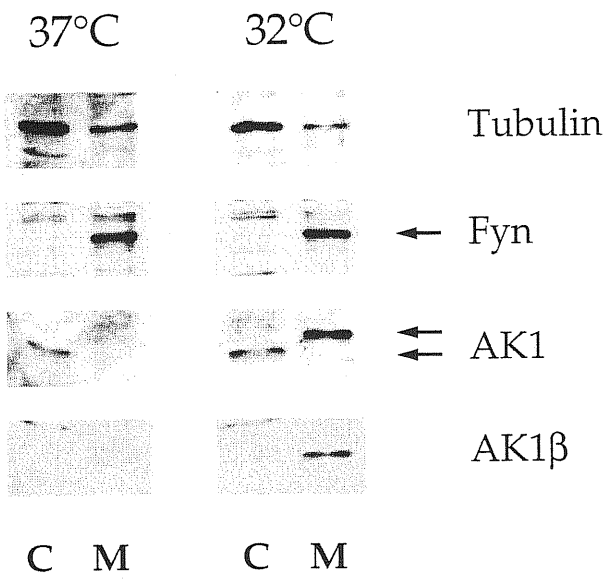


Figure 5
Biochemical localisation of endogenous AK proteins in Val5.
Immunoblotting of purified cytoplasmic (C) and membrane (M) fractions from Val5 cells grown at 37°C or kept for 24 hrs at 32°C (see Materials and Methods). Equal amounts of lysates from cells growing at the two temperatures were separated by SDS/PAGE, transferred to nitrocellulose, and stained with the indicated antibodies. Cytoplasmic and membrane fractions are not comparable with each other because of a different dilution.

To biochemically confirm the differential localisation of the AK1β protein, a fractionation was performed to separate cytoplasmic from membrane associated proteins. Briefly, cells were collected in isotonic buffer, lysed by sonication, and fractionated by centrifugation on a sucrose gradient. Cytosolic and membrane fractions were subsequently analysed by immunoblotting with the anti-AK1 and the AK1β-specific antisera. As can be observed in Figure 5, AK1β specifically segregated with the membrane fraction. To confirm purity of the fractions, endogenous Fyn, which is associated to the membrane via N-myristoylation [121], and Tubulin, which is largely cytoplasmic, were analysed in the same samples. Altogether the reported data demonstrated that AK1β is localised to the plasma membrane, as could be inferred from its primary sequence.

Evidence for an involvement of AK1β in wtp53-dependent cell-cycle arrest

To evaluate the biological relevance of AK1β induction in the context of p53 function, I used an antisense mRNA approach in the Val5 cell line as described in [44]. Expression vectors containing the 3'UTR region of AK1 in either sense or antisense orientation were microinjected in Val5 cells at 37°C, along with a vector expressing the human transferrin receptor (hTR) as a marker to score injected cells. After microinjection, the cells were shifted to 32°C and the percentage of cells in S-phase was measured by BrdU incorporation after 22hrs (Figure 6A). At this time point Val5 cells undergo p53-dependent cell cycle arrest and only 15-20% of cells are in S-phase, as compared to the average 50% of S-phase cells detected at 37°C (see Figure 6B). Cells injected with the antisense construct (AS) arrested less efficiently, displaying a much higher proportion of S-phase than background uninjected cells (Figure 6B). This difference is less evident at longer times, and by 48 hrs at 32°C, both injected and uninjected cells are essentially arrested (not shown).

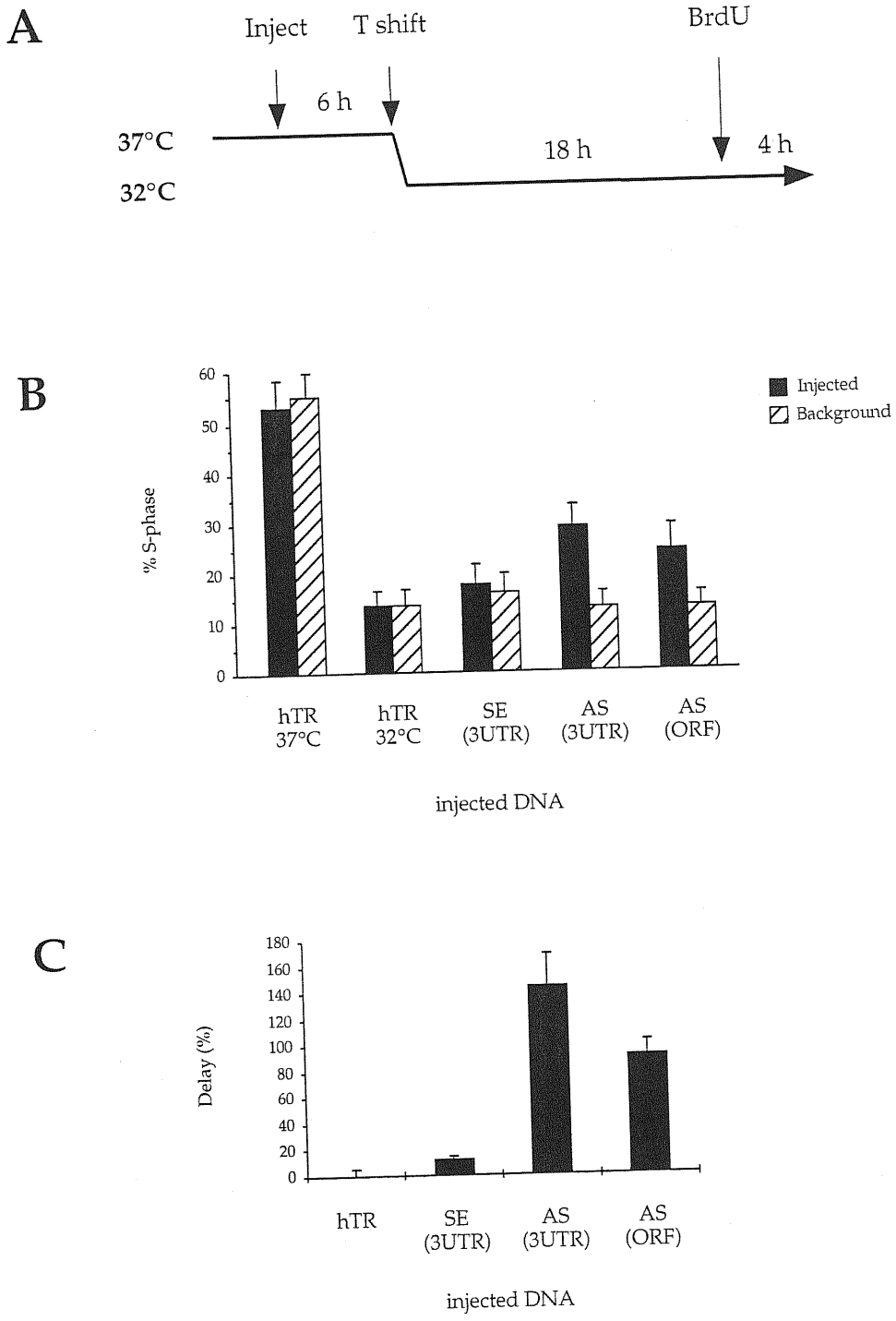


Figure 6
Expression of antisense AK constructs delays p53-induced growth arrest.
(A) Schematic rapresentation of the experimental protocol. Cells were microinjected at 37°C with 50 ng/µl of the indicated plasmids together with 50 ng/µl of pGDSV7-hTR as a marker. Cells were shifted to 32°C after 6 hrs, and fixed after 22 hrs. BrdU was added for the last 2 hrs to analyse the % of S-phase cells. (B) Effect of antisense AK expression on Val5 cell-cycle arrest at 32°C. Cells were microinjected with the 3'UTR of AK1 in sense or antisense orientation, or with a fragment corresponding to the ORF of AK1 in antisense orientation (constructs are schematically shown in Figure 7A). Data relative to microinjection of hTR in Val5 cells kept at 37°C or shifted at 32°C are also included. At least 80 cells were scored for each microinjection, and the results are the mean of at least three independent experiments. (C) Quantification of the interference with p53-dependent arrest exerted by various constructs. Values are expressed as Delay (%) = (% S-phase of injected cells - % S-phase of background cells)/% S-phase background cells.

The extent of interference in cell-cycle arrest can be expressed as Delay % (i.e. percent S-phase of injected cells minus percent S-phase of uninjected cells, divided by percent S-phase of uninjected cells) as shown in Figure 6C. Similar results, albeit less striking, could be obtained using the coding region of AK1 in antisense orientation (ORF). Injection of hTR alone had no effect, as well as injection of the 3'UTR of AK1 in sense orientation (SE). It is worth noting that similar results could be obtained using an antisense construct to Waf1 under the same experimental conditions [44]; thus, it appears that expression of antisense AK1 interferes with wtp53 induced cell-cycle arrest in Val5 cells.

Co-expression of AK1β neutralizes the effect of antisense 3'UTR constructs

In the present work I provide evidence that, at least in the Val5 cell system, the protein actually induced by p53 is AK1β, while cytoplasmic AK1 is constitutively expressed at detectable levels. Since antisense 3'UTR constructs are complementary to both AK forms, I reasoned that antisense mRNAs were more likely interfering with AK1β induction, rather than depleting cytoplasmic AK1. To test this hypothesis, I decided to evaluate the ability of each AK protein to neutralise the effect of expression of antisense 3'UTR. Therefore, triple co-injections were performed in which expression vectors encoding VSV-tagged versions of either AK1 or AK1β were microinjected together with the antisense 3'UTR construct, and the hTR marker. It is important to point out that there was no overlap between the coding regions and the antisense 3'UTR fragment used (see Figure 7A). As shpwn in Figure 7B, co-expression of cytoplasmic AK1 had no consequence on the effect of antisense 3'UTR, and injected cells still displayed defective cell-cycle arrest. On the contrary, co-expression of AK1β neutralised the effect of antisense 3'UTR, and injected cells arrested as efficiently as background uninjected cells. Indirect immunofluorescence confirmed that the AK proteins were actually expressed within injected cells (not shown).

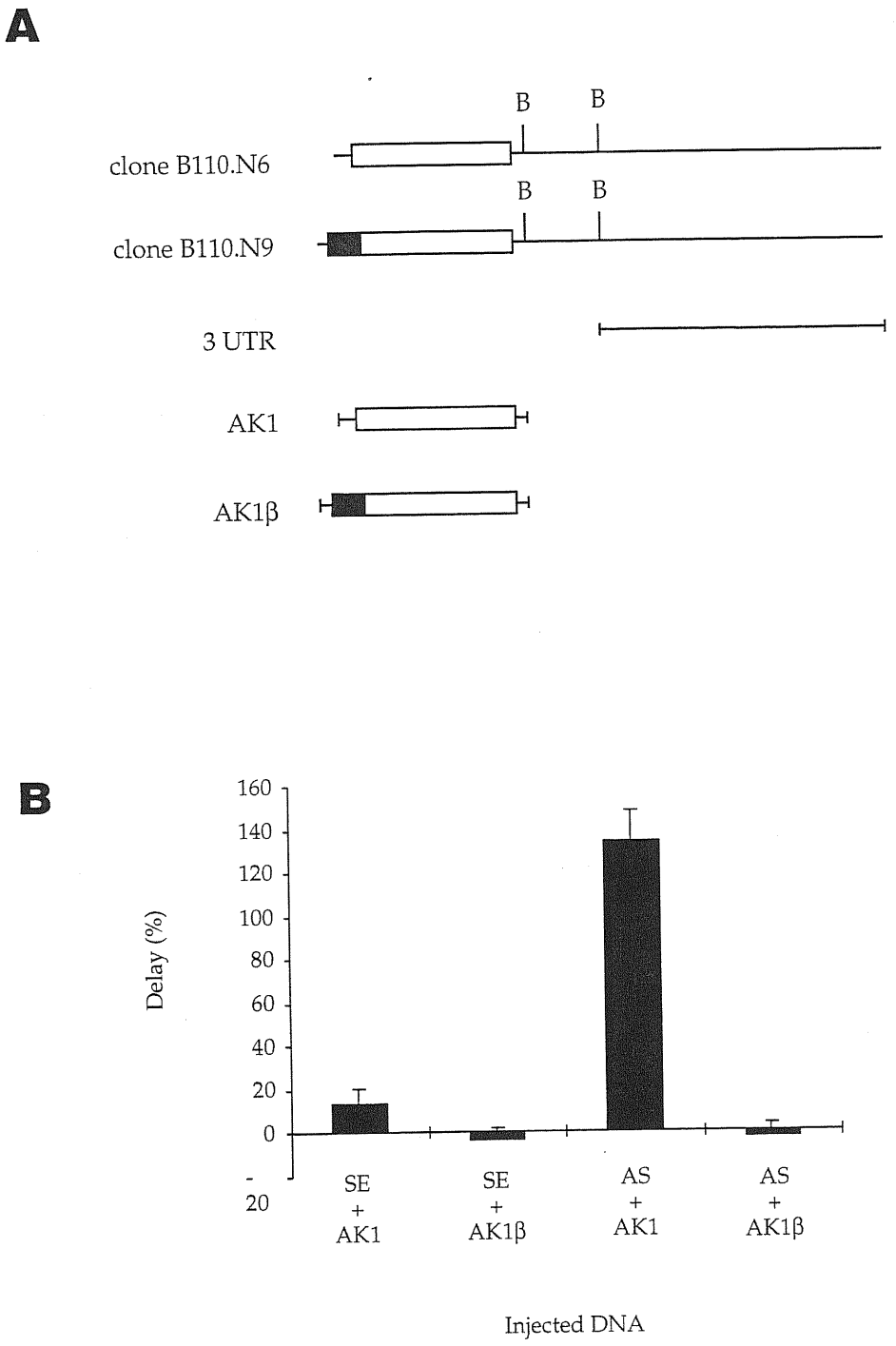


Figure 7
Coexpression of AK1β can revert the effect of antisense constructs.
(A) Structure of the cDNAs corresponding to AK1 and AK1β, in relation to the constructs employed in the antisense experiments. (B) Result of co-expression of AK1 or AK1β on the effect of antisense AK constructs in Val5 cells. Plasmids expressing the VSV tagged versions of either AK1 or AK1β were co-injected at 50 ng/µl with the 3'UTR construct in either sense or antisense orientation as described in the legend to Figure 6. Results, expressed as Delay(%), are the mean of at least three independent experiments in which more than 80 injected cells were analysed.

Overexpression of either AK1 or AK1β alone had no effect on the cell-cycle of Val5 cells at 37°C (not shown). Co-expression of either AK1 or AK1β together with the 3'UTR in sense orientation did not influence the cell-cycle arrest of Val5 cells at 32°C (Figure 7B). These results strongly imply that the delay in p53-induced arrest observed in Val5 cells upon expression of antisense AK constructs is the consequence of blocking p53-dependent induction of the alternative AK1β protein.

The alternative form of AK1 mRNA is induced by p53 in human cells

The human gene for cytosolic Adenylate kinase has been sequenced and characterised [159]: it is composed of 7 exons spanning a region of about 12 Kb on chromosome 9 q34.1-q34.2. Through a computer based analysis of the human AK1 gene, a number of putative p53 binding sites could be identified, both within the promoter and intronic sequences ([17] and J.-C. Bourdon, personal communication). These elements are detailed in Table 1, and their positions within the human AK1 gene are indicated in Figure 8A. There is a cluster of degenerated decameric sites in the promoter, at approximately 500 bp from the transcription start site; two putative p53-responsive elements are located within the first intron; and another cluster of decamers is located in the second intron. The presence of putative p53 responsive elements in the human AK1 gene provides a conceptual support for the observed p53-dependent regulation of the murine gene; moreover, it strongly implies that also human AK1 might be regulated by p53. Within intron 2, a region could be identified having high similarity to the alternative 5' sequence of clone B110.N9; this region is indicated as exon IIβ in Figure 8A. This sequence is surrounded by consensus sites for splicing (not shown). Notably, if this putative exon IIβ was joined to exon 3, an alternative form of human AK1 would be generated, perfectly corresponding to murine AK1β.

Indeed, computer search of the dbEST database identified a human cDNA (EST clone 781374, Acc.N. AA430294) which exactly corresponded to the hypothesised alternative transcript, thus confirming its existence in human cells. Figure 8B shows the N-terminal alignment of the deduced murine and human peptides: although the primary sequences are not identical, the residues involved in putative myristoylation are strikingly conserved, suggesting that EST clone 781374 might be the human homologue of murine AK1β.

TABLE1: Putative p53-binding sites identified in the human AK1 gene

Site *	*Pos.***	*Sequence*
1.	(-495)	GGTCCTGGCC AGGGCCTCT GGGCAGGCTC TGA GGGCGGGCTT GG GGACTGTGG C CAGCAaAGCCC
2.	(+524)	GGGCTTGCCT T GGCCTTGTCT
3.	(+1169)	CAGCAaAGCCA CTT CAGCTGCTA CTGGGTGCCA GGCCCAGCGT AGCAGAAACT CAG GGCCGAGTTG GACCC TAGCTGGCCC TC GAACCAGTCT
4.	(+3994)	GGACGAGACA GAGGTTGG AGCCAAGCTC ACCGCCCTCTCT GGGCAAGAGT T CCACATGCCC

* Sites are numbered as in Figure 6A. ** The position of each sequence is indicated with respect to the transcriptional start site (+1)

To evaluate if the alternative form of AK1 is regulated by wtp53 in human cells, I analysed expression of AK1β by semi-quantitative RT-PCR in two different cell lines carrying a temperature sensitive allele of either murine or human p53 (see legend to Figure 8). An upper primer was designed on exon IIβ and a lower primer was designed within exon 6 to yeld a specific product of 400bp. Amplification products were Southern-blotted and detected using a specific internal probe. Regulation of AK1β mRNA was then compared to regulation of GAPDH amplified from the same cDNA. As shown in Figure 8C, the transcript corresponding to the alternative AK1β protein was selectively detected only at the permissive temperature in both cell-lines. To extend this observation to a more physiological system, expression of AK1β was also analysed in human fibroblasts after exposure to UV irradiation, under conditions known to efficiently activate p53 function (M. Monte, unpublished results). Accordingly, AK1β was significantly induced by UV treatment, both in embryonic fibroblasts and IMR90 cells (Figure 8C). Altogether, these data demonstrate that a human AK1β transcript exists, and suggest that such transcript is regulated by wtp53 similarly to what has been observed for the murine gene.

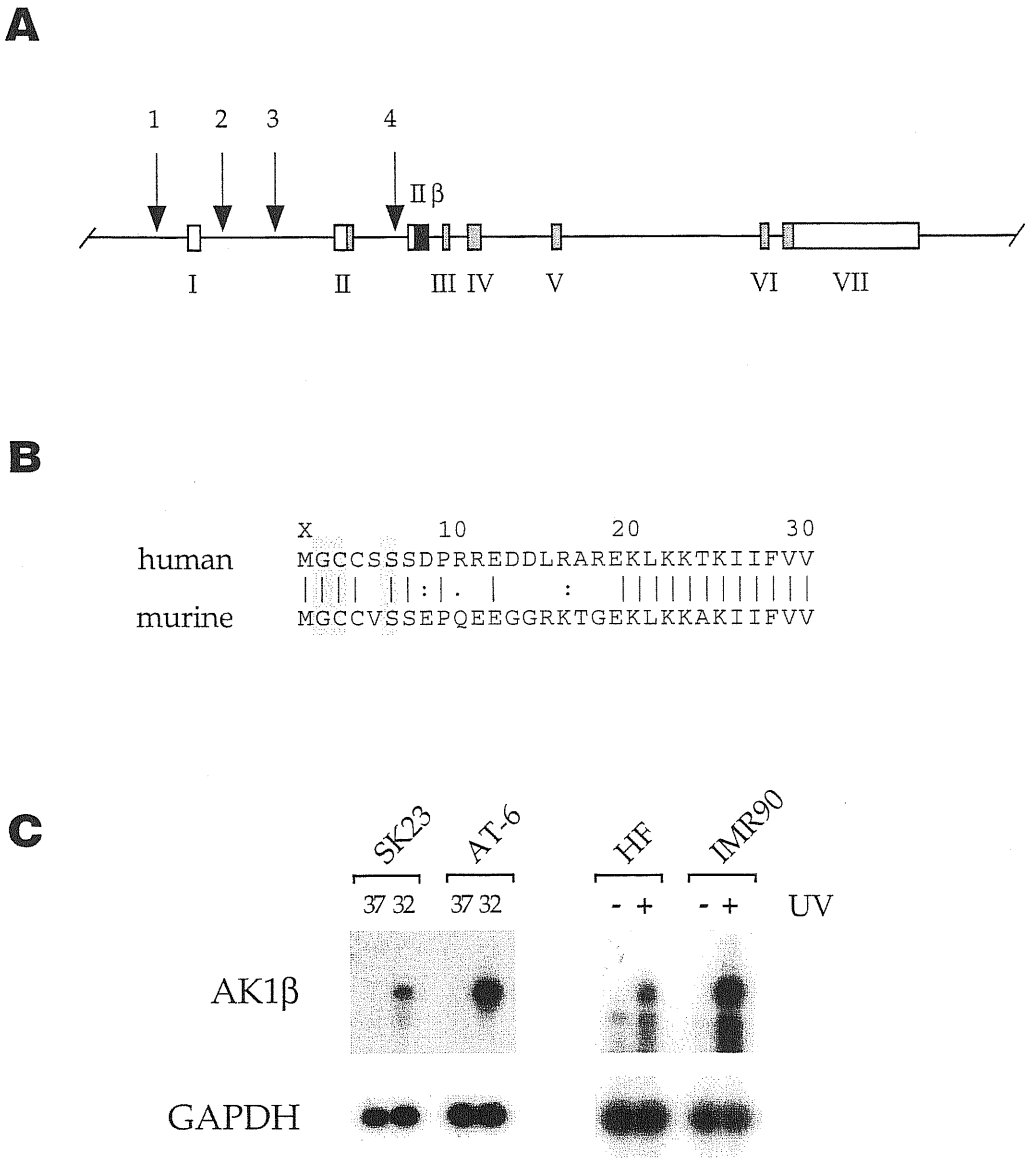


Figure 8
An alternative AK1 mRNA is also induced by wtp53 in human cells.
(A) Schematic structure of the human AK1 gene. Arrows indicate the four putative p53-binding sites (see Table1). Exons are numbered according to Matsuura et al. (1989), and translated sequences are shaded. Exon IIβ corresponds to the alternative 5' sequence of the putative human AK1β. (B) Sequence alignment of the N-termini of murine and human AK1β proteins. Shaded boxes indicate conserved residues involved in myristoylation (see text). (C) Semi-quantitative RT-PCR analysis of human AK1β expression in human cells. SK-23 are an ovarian cancer cell line transfected with the Val135 ts mutant of murine p53 (Vikhanskayaet al., 1994). AT-6 are U2OS osteosarcoma cells, transfected with the Val143 ts mutant of human p53 (Buckbinder et al., 1994). At 32°C the exogenous p53 in these cells assumes a wt conformation and induces expression of target genes. Expression of human AK1β was also analysed after 10J/m2 UV irradiation in human embryonal fibroblasts (HF) and IMR90 cells. Identical aliquots of random primed cDNA were used for PCR amplification with primers specific for human AK1β or GAPDH. Reaction products were analysed by Southern blotting with specific probes.

DISCUSSION

In the present study I have reported that one of the six p53-inducible transcripts identified by Renè Utrera in Val5 cells encoded a novel, alternative form of cytosolic Adenylate Kinase (AK1, EC 2.7.4.3), differing from AK1 for the addition of 18 extra aminoacids at the N-terminus. I named this alternative product AK1β and I showed that in Val5 cells the AK1β protein is specifically upregulated upon wt p53 activation.

A number of observations suggest that AK1β might be a direct and specific transcriptional target of p53: first, upregulation of the transcript required a transcriptionally competent p53; second, efficient induction of the mRNA was observed in Val5 cells even in the absence of protein neosynthesis; and finally, AK1β mRNA was upregulated in a wt p53-dependent manner in mouse fibroblasts exposed to UV irradiation. An important additional support to this inference was provided by analysis of the human AK1 gene: in fact, a number of consensus p53-binding sites are located in the promoter and first introns of human AK1 (see Figure 8), and some of them may be conserved in the murine gene. Importantly, I showed that a human transcript corresponding to AK1β indeed exists, and is specifically upregulated by wtp53 in human cells. This last observation has two relevant implications: it demonstrates that at least one of the putative p53-responsive elements is functional, confirming the validity of the sequence-based predictions, and it demonstrates that p53-dependent expression of AK1β is evolutionary conserved, supporting its possible biological significance. A detailed molecular analysis will be required to clearly identify the functional p53-responsive elements in the AK1 gene, and to define the molecular basis for expression of the alternative AK1 transcript. With respect to this last point, it will be important to define whether AK1β is produced by alternative splicing of exon IIβ, or is transcribed from an alternative promoter within intron 2, similarly to what has been reported for murine MDM-2 [9].

The extra aminoacids at the N-terminus of murine and human AK1β contain a consensus signal for myristoylation, a lipid modification known to mediate protein targeting to the cell membrane [201]. Accordingly, I could show by immunofluorescence that exogenous AK1β localises to the plasmamembrane. By biochemical analysis, I confirmed that endogenous AK1β segregates with the membrane fraction. In most myristoylated proteins, such as src-family members (except Src itself and Blk) and Gα subunits of heterotrimeric G-proteins, membrane association is stabilised by attachment of palmitate to the thiol group of N-terminal cysteine residues, reaction which is subordinated to co-translational addition of myristate on Gly 2 [14, 121, 201]. Given that N-terminal cysteine residues are present at appropriate positions and are conserved in both murine and human AK1β proteins (see Figures 4 and 8), it is possible that membrane association of AK1β is also reinforced by palmitoylation.

Addition of extra aminoacids at the N-terminus should not dramatically affect the substrate binding region of Adenylate Kinase. In fact, crystallographic data on the structure of porcine cytosolic AK1 (extremely similar to both human and murine AK1) indicate that the N-terminus and the C-terminus point to the outside of the molecule [53, 215]. This suggests that AK1β should maintain ATP:AMP phosphotransferase activity.

The biological relevance of AK1β induction in the context of p53-dependent growth-arrest was suggested by antisense ablation experiments in Val5 cells. Specifically, expression of an antisense mRNA corresponding to the 3'UTR of AK1 (and AK1β) significantly delayed p53-induced cell-cycle arrest of Val5 at 32°C, and this effect could be efficiently reversed by co-expression of the AK1β protein, but not by cytoplasmic AK1. Thus, AK1β should acquire some novel function with respect to AK1, and this function is probably related to p53-modulated biochemical events determining cell-cycle arrest.

A first hypothesis to tie up AK1β biochemical activity with its biological effect is that p53-dependent upregulation of Adenylate kinase serves to provide ATP backup to energy-consuming processes such as DNA repair or apoptosis. However, I do not favour

such hypothesis since the effect of antisense 3'UTR could not be reverted by coexpression of even high amounts of cytosolic AK1, indicating that a function exclusive to AK1β is required. Nevertheless, it is still possible that membrane targeting serves to locally increase AK activity at sites where it might be specifically required. In bacteria, for example, a fraction of AK1 is membrane-associated and is involved in energy consuming activities such as phospholipid neo-synthesis, or transport of osmoprotectants during osmotic shock [85, 248].

A second hypothesis is that re-localisation of AK1 to the membrane compartment, or the mere addition of extra aminoacids at the N-terminus, could modulate the substrate specificity of the enzyme. It is in fact known that AK1 can complement nucleoside diphosphate kinase (NDPK) deficiency in bacteria [145], and mammalian AK1 has full NDPK activity and can efficiently phosphorylate all four ribo- or desossi- nucleosides diphosphates [145]. In *Pseudomonas aeruginosa*, under certain growth conditions, cytoplasmic NDP kinase is proteolytically converted to a membrane associated form which acquires a strong specificity for synthesising GTP in preference to other NTPs [216]. Similarly, AK1β could have a more selective substrate specificity than AK1. Crucial events take place at the inner face of the membrane, where signals coming from the external environment are integrated in multiple transducing pathways, regulating processes such as cell-division, cell-differentiation, or cell-death (see [107, 239] and references therein). Many important signalling events involve protein phosphorylation or GTP hydrolysis and it is plausible that the local concentrations of adenine and guanine nucleosides di- or three- phosphates influence such processes; AK1β might therefore regulate homeostasis of ATP or GTP at the sub-membrane compartment.

A third hypothesis is that re-localisation of AK1 to the membrane compartment facilitates functional interaction with other proteins. Notably, in Gα proteins the N-terminal anchor participates in interaction with the Gβγ subunits [33, 125]. It is therefore tempting to speculate that in AK1β the extra aminoacids at the N-terminus might create a docking site for protein-protein interactions. Furthermore, AK1 protein is known to

undergo significant conformational changes during the catalytic cycle (see [53, 77] and references therein). This conformational switch might transduce a signal to hypothetical interacting proteins, similarly to what happens with G proteins and small-GTPases [89, 102]. It is worth noting that a possible effect of p53 on signal transducing pathways has already been hypothesised; in fact human p53 induces the RGS14 member of the RGS family of regulators of G-protein signalling [122]. RGS14 interacts with Ga subunits and inhibits G-coupled growth-factor receptor mediated activation of mitogen-activated kinases [23].

A final hypothesis is that addition of the extra residues and consequent re-localisation to the membrane compartment might provide to AK1β a novel biochemical function, distinct from that of ADP kinase, such function being involved in p53-dependent cell-cycle arrest. In this regard, it is worth noting that the Nm23 metastasis-suppressor genes encode NDP kinases (see [38, 61] for review), and that a few reports suggest that NDPK activity is dispensable for their metastasis suppressing function [62, 129, 148]. A recent study revealed that Nm23-H1 forms a complex with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) *in vivo*, and such complex has ser/thr-specific protein-phosphotransferase activity [61]. Although the relevance of this novel enzymatic activity for the metastasis-suppressing function of Nm23-H1 is still to be assessed, it provides a remarkable example of an "old" enzyme acquiring a "novel" function.

In conclusion, Val5 cells are an excellent model for studying the cell-cycle arrest function of the p53 tumor suppressor. Within this cellular system I have observed the involvement of a membrane-associated form of Adenylate Kinase in a growth-regulatory pathway activated by p53. Future work will be required to extend the possible relevance of AK1β expression to other cellular systems, and to test the various aforementioned hypotheses on AK1β function in the context of p53 activity.

PART 4

A PROLINE-RICH MOTIF IN p53 IS REQUIRED FOR TRANSACTIVATION-INDEPENDENT GROWTH ARREST AS INDUCED BY GAS1

In addition to its fundamental activity as a transcription factor, a number of observations indicate that important aspects of p53 function might be independent from its sequence-specific transactivation activity. These aspects are much less-characterised than those involving regulation of gene expression.

By studying the effect of overexpression of the growth-arrest-specific gene Gas1 in transformed cell lines, it was discovered that growth-suppression by Gas1 occurred only in cells containing a functional p53 [42]. Subsequently, it was demonstrated that sequence-specific transactivation by p53 was not required for mediating Gas1-induced growth-suppression, since p53 alleles mutated in the transactivation domain still supported full inhibition by Gas1 [45].

I have been collaborating with Elisabetta Ruaro in a research project which was aimed at defining the functional domains of p53 required to mediate Gas1-induced growth-suppression. We observed that wild-type conformation of the DNA binding domain was necessary but not sufficient for cooperation with Gas1. Interestingly, we could demonstrate that Gas1-mediated growth-arrest required the proline-rich domain of p53 (see PART 1). On the basis of competition experiments we proposed that cooperation with Gas1 involves interaction of a still unknown cellular protein with the poly-proline domain of p53.

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A proline-rich motif in p53 is required for transactivation-independent growth arrest as induced by Gas1

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Contributed by Arnold J. Levine, February 20, 1997

ABSTRACT **The involvement of p53 in regulating diverse cellular processes dictates that it must respond to multiple signaling mechanisms, thus coordinating the response to various "stress conditions." Genotoxic stress has served as a paradigm to dissect the transactivation-dependent branch of the pathway by which p53 can induce growth arrest. Alternate mechanisms have been invoked to explain transactivation-independent effects of p53, especially in the context of apoptosis. We have identified a p53-dependent pathway initiated by the gas1 product, a plasma membrane protein highly expressed during G0, which activates a transactivation-independent p53 growth arrest function. Through a detailed deletional analysis and site-specific mutagenesis of p53 we show that the Gas1-dependent signal transduction relies on a proline-rich region (amino acids 63–85) of murine p53. *In vivo* competition experiments using combinations of such mutants implicate this functional domain of p53 as a docking site in the transmission of antiproliferative signals.**

Wild-type (wt) p53 can mediate a variety of antiproliferative effects, such as induction of G1 and G2 arrest, activation of apoptotic cell death and suppression of oncogene-mediated transformation (1–3). Four structural and functional domains have been identified in the p53 protein and some of them are required and sufficient for specific functions (4). Genotoxic stress as induced by various types of chemical and physical DNA damaging agents, has been the most studied and used inducer of p53 functions. Accumulating evidence indicates that p53 can respond to other stress stimuli that do not appear to induce, at least directly, DNA damage. Hypoxic conditions (5), heat shock (6), calcium phosphate treatment (7), or ribonucleotide depletion (8) have all been reported to activate p53 functions. A common event in the response to such stress stimuli is that the cellular levels of p53 dramatically rise, mainly due to posttranslational stabilization of the protein (3, 9, 10). In normal fibroblasts the commonly observed consequence of enhanced p53 levels, as induced by DNA damaging agents, is G1 arrest that presumably allows repair of the DNA damage before entry into S phase (4, 11). The sequence-specific transactivation function of p53 has been shown to be instrumental in establishing G1 arrest and among the genes induced by p53 the most important candidate to mediate cell cycle block is p21/waf1 (12–14). Increased levels of p21/waf1 have been shown to interfere with the cell cycle machinery through the combined inhibition of the cyclin/cdk kinases and the replicative functions of the DNA polymerase δ-associated factor PCNA (15, 16). However, p21/waf1 deficient mice (17, 18) retain p53-dependent G1 arrest function, albeit compromised relative to the normal controls. These results suggest the existence of yet another p53-dependent G1 arrest pathway that either requires transactivation of other genes or else operates through a different function and perhaps a different domain of p53.

It has been widely described that p53 is able to directly interact with a multitude of cellular and viral proteins (4). Therefore, it may well be possible that an alternative domain of p53 is responsible for a specific interaction with a yet uncharacterized cellular protein, which mediates the growth suppressing function of p53. Involvement of such an alternative function has particularly been implicated in the case of p53-dependent apoptosis (2). In fact, although in some cases (19, 20) p53 mutants unable to transactivate are also completely deficient in apoptosis, other reports indicate that the sequence-specific transactivation function is either dispensable or at least not fully sufficient (21–23).

Nontransformed fibroblasts in culture respond to growth factor deprivation by exiting the cell cycle and entering a reversible growth arrest or G0. This state is marked by the increased expression of the gas (growth arrest specific) genes that become rapidly downregulated upon readdition of growth factors. The function of gas genes has been linked to the regulation of apoptosis at various levels (24–26) and in the case of Gas1 to growth suppression (27, 28). When Gas1, a plasma membrane glycoprotein, is ectopically expressed it blocks the G0 to S transition of quiescent fibroblasts and the cell cycle progression in various types of asynchronously growing cells (27, 28). We have demonstrated that this effect of Gas1 requires wt p53 but is independent of its transactivation function (29). The aim of this work was therefore to elucidate the p53 domains that are essential for mediating Gas1 function to gain new insights into how a membrane associated protein could exert its suppressive function via p53.

MATERIALS AND METHODS

Plasmids. wt murine p53 cDNA was excised from pMSVcL vector (30) and subcloned in pGDSV7 under the control of simian virus 40 early promoter (31) to generate pGDSV7-mp53. Numbering of murine p53 amino acids is done according to Soussi *et al.* (32), assuming that the second in-frame ATG is used as initiation codon. Deletion Δ(8–63) was generated by digestion of pGDSV7-mp53 with *Eco*RV–*Sac*I, elimination of protruding ends with T4 DNA polymerase and re-ligation. Deletion Δ(8–106) was created by insertion of an oligonucleotide adaptor between the *Eco*RV and *Pst*I sites in mp53. Deletion Δ(8–85) was produced by inserting a PCR amplified

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Abbreviation: wt, wild type.

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fragment corresponding to amino acids 86–106 in the *Eco*RV site of the Δ(8–106) construct. Deletion M154 was created by fusion of the two *Nco*I sites corresponding to Met-1 and Met-154 by *Nco*I digestion, removal of the fragment and religation. Deletion Δ(8–63)dl330 was generated by assembling the truncated C terminus from pCMVdl330 (33) as a *Kpn*I–*Xba*I fragment in the Δ(8–63) construct. Plasmid p53wt-5980 was generated by PCR-based directed mutagenesis, on the background of plasmid pCMVp53wt (34). Construct Δ(8–63)/5980 was created by replacing the *Pst*I fragment spanning amino acid 64 to 109 in the Δ(8–63) mutant with the corresponding fragment derived from p53wt-5980 and clone Δ(8–63)dl330/5980 was generated by assembling the N terminus from Δ(8–63)/5980 as an *Xho*I–*Kpn*I fragment on the Δ(8–63)dl330 construct. All constructs were sequenced to confirm the introduced changes using an automated DNA sequencer (ALF–Pharmacia).

***In Vitro* Translation and Electrophoretic Mobility-Shift Assay.** The expression vectors containing the mp53 deletion constructs were directly used for *in vitro* transcription/translation, using the TNT T7 Coupled Reticulocyte Lysate system (Promega) according to the manufacturer's instructions. DNA-binding reactions were performed in a volume of 15 μl: *in vitro* translated proteins were incubated with ≈10^5 cpm of ^{32}P-labeled, annealed p53CON oligonucleotide (5′-GGACATGCCCGGGCATGTCC-3′) (35), in binding buffer (10 mM Tris·HCl, pH 8/100 mM NaCl/1 mM EDTA/8 mM spermidine/1 mM DTT/5% glycerol/0.2 μg/μl BSA/100 ng salmon sperm DNA) at room temperature for 20 min. All the reactions contained 2 μl PAb421 hybridoma supernatant. Complexes were loaded on 4.5% nondenaturing polyacrylamide gel and electrophoresed at 4°C. A sheet of plastic foil was placed between dried gel and autoradiographic film to cut out the signal from [^{35}S]methionine. Specificity of the binding was confirmed by competition with excess cold p53CON or p53MUT (5′-GGACAGTCGGCCGACTGTCC-3′) oligonucleotides.

Microinjection and Immunofluorescence. Cells cultured in DMEM containing 10% fetal calf serum were grown on coverslips (5×10^3 cells per cm^2) in 35-mm diameter Petri dishes. After 24 hr of incubation at 37°C in 5% CO_2 atmosphere, cells were microinjected by the Automated Injection system (Zeiss) as described (27). For expression analysis they were incubated for 12 hr and then processed for immunofluorescence. For growth inhibition assay, after injection cells were left for 24 hr in DMEM supplemented with 10% fetal calf serum. DNA synthesis assay was performed by adding 50 μM BrdUrd to the culture medium 6 hr before fixation. Cells were fixed and processed for immunofluorescence as described (27). mp53 was revealed by using the PAb240 antibody followed by goat anti-mouse IgG1-fluorescien isothiocyanate (Southern Biotechnology Associates); BrdUrd was revealed by mAb anti-BrdUrd (Amersham) followed by goat anti-mouse IgG2a-rhodamine isothiocyanate (Southern Biotechnology Associates). p21/waf1 was revealed by the affinity purified antibody described in Del Sal *et al.* (29) followed by goat anti-rabbit RITC (Southern Biotechnology Associates).

RESULTS

To identify the p53 domains that are essential for mediating the Gas1 induced growth arrest, a series of deletion mutants was generated from wt murine p53 (see *Materials and Methods*) as shown in Fig. 1*a*. All mutants, except Met-154, are internal deletions in which the original translation start site has been conserved. Analysis of the respective *in vitro* translation products by SDS/PAGE confirmed their expected size (Fig. 1*b*). We next tested the ability of the p53 deletion mutant products to form gel-stable complexes with the p53CON oligonucleotide by a mobility retardation assay (electrophoretic mobility-shift assay) as shown in Fig. 1*c*. Mutants with small N-terminal deletions [Δ(8–63) and Δ(8–85)] specifically bind to the

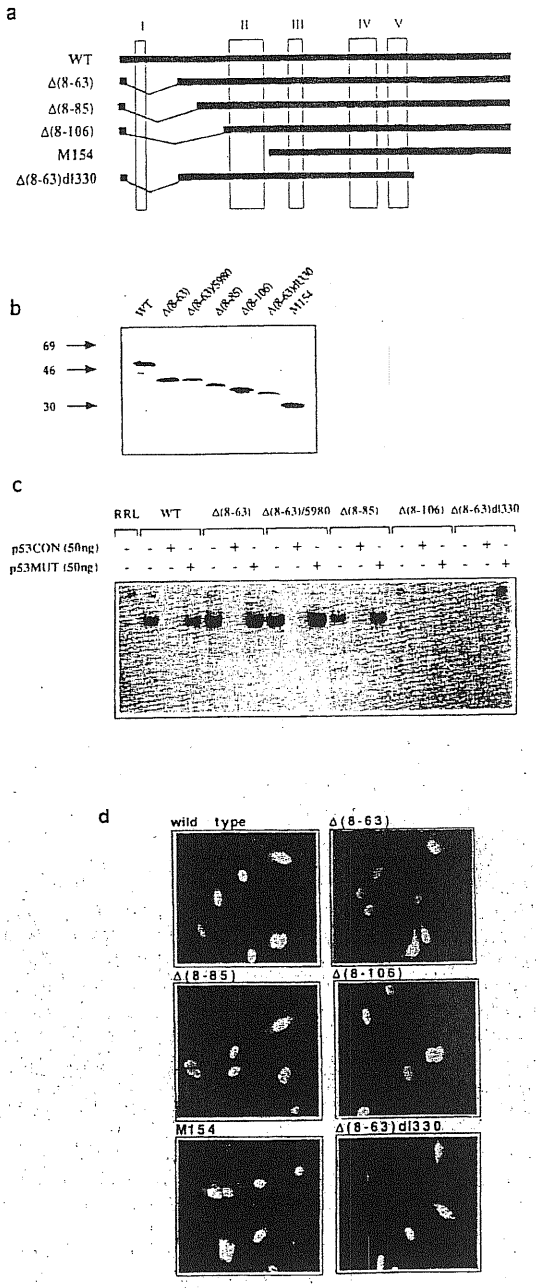

FIG. 1. Expression, sequence specific DNA binding activity, and nuclear localization of murine p53 deletion mutants. (*a*) Schematic representation of the p53 mutants used. Numbering of murine p53 amino acids is done according to ref. 32. (*b*) *In vitro* translated murine p53 mutants were analyzed by SDS/PAGE to confirm the expected apparent molecular weights. The structure of deletion Δ(8–63)/5980 is described in Fig. 3. (*c*) *In vitro* translated proteins were tested for sequence specific DNA binding by electrophoretic mobility shift assay with the p53CON oligonucleotide as a probe. Specificity of the binding was confirmed by competition with excess cold p53CON or p53MUT oligonucleotides. (*d*) 100 ng/μl of each pGDSV7 expression plasmid (31) containing the different p53 deletion mutants, was microinjected into the nuclei of BALB/c (10)1 cells (36). p53 protein expression was analyzed 12 hr later by indirect immunofluorescence using PAb240. Images were obtained with a LSM 450 confocal microscope (Zeiss).

consensus sequence similarly to wt p53, and this binding is competed by addition of excess specific oligo. Larger N-terminal deletion mutants [Δ(8–106) and Met-154] are severely compromised in DNA binding activity, as is also noticed for the amino/carboxyl double deletion lacking the oligomerization domain [Δ(8–63)dl330].

To evaluate the subcellular localization and expression level of the p53 deletion mutants, each expression vector was microinjected in BALB/c (10)1 (p53 null) fibroblasts (36). The mutant proteins accumulated into the nucleus similarly to wt p53 as assessed by immunofluorescence (Fig. 1*d*).

Next, the mutants were analyzed for their ability to induce growth arrest in BALB/c (10)1 fibroblasts. Ectopic expression of the various mutants was performed by microinjection, and its effect on cell cycle was evaluated by measuring the relative level of BrdUrd incorporation (S-phase). All the p53 mutants were defective in inhibiting DNA synthesis, whereas wt p53 exhibited a strong inhibitory effect (Fig. 2*A*). As previously shown (29), expression of Gas1 alone is also unable to inhibit DNA synthesis in these cells (Fig. 2*B*). Next, we evaluated if any of the described p53 mutants was able to reconstitute the Gas1-dependent growth arrest pathway; this was done by coexpressing Gas1 with the various deletion mutants (Fig. 2*B*). Only mutant Δ(8–63) was able to cooperate with Gas1 to inhibit DNA synthesis to a similar extent as obtained when Gas1 is overexpressed in cells harboring endogenous wt p53 (27, 28). The Δ(8–63) p53 mutant lacks the entire transcriptional activation domain indicating that not only the transactivation function but the structural domain itself is dispensable for mediating Gas1 induced growth arrest. From Fig. 2*B* it can also be deduced that integrity of the DNA binding domain is critical for cooperation with Gas1. In fact, deletions affecting DNA binding activity [Δ(8–106) and Met-154] are also defective in the ability to reconstitute Gas1 induced growth suppression (Fig. 2*B*). In addition the double deletion Δ(8–63)dl330, also lacking the oligomerization domain, loses the ability to cooperate with Gas1, in line with the observation that efficient DNA binding requires the assembly of p53 into homotetramers (37–39). This is in line with our previous results showing that point mutations affecting the DNA binding domain, Val-135 (at 37°C) and KH215, are not able to cooperate with Gas1 (29).

Most significantly, we could demonstrate that sequence specific DNA binding activity per se is not sufficient to reconstitute the Gas1 growth suppression pathway. In fact, in stark contrast to the Δ(8–63) deletion, mutant Δ(8–85) is completely defective in reconstituting the Gas1 induced growth arrest (Fig. 2*B*). Similarly to Δ(8–63), mutant Δ(8–85) retains both wt conformation and sequence-specific DNA binding (Fig. 1*c*). This last observation strongly suggested that the region between amino acids 63–85 could represent a new functional domain of p53 that is critical in mediating the Gas1-dependent growth arrest. The region of murine p53 between residues 63–85 contains several prolines (Fig. 3*a*). In particular, the PXXP motif has been described as a docking site for interaction with SH3 domains (40, 41). The corresponding human p53 sequence contains five such motifs while murine p53 contains only two, the first one being conserved between human and mouse. To analyze whether the prolines contained in this region could be responsible for cooperation of p53 with Gas1, point mutations were generated by replacing Pro-76, -79, and -81 with alanines, and Pro-84 with serine. The mutations were introduced into both full-length p53 (p53wt-5980) and N-terminal truncated p53 (Δ(8–63)/5980) (Fig. 3*a*). Both mutant proteins localized to the nuclei of microinjected BALB/c (10)1 cells (Fig. 3*b*). Since Δ(8–63)/5980 retains sequence specific DNA binding (Fig. 1*c*), we evaluated the ability of the mutants to exert sequence specific transactivation by measuring the induction of p21/Waf1 protein in BALB/c (10)1 cells. The p53wt-5980 mutant, retaining the transactivation domain, induced efficient expression of endogenous p21/Waf1, while the transactivation deficient Δ(8–63)/5980 mutant completely lacked such activity (Fig. 3*b*). As expected from this observation, the growth inhibitory activity of

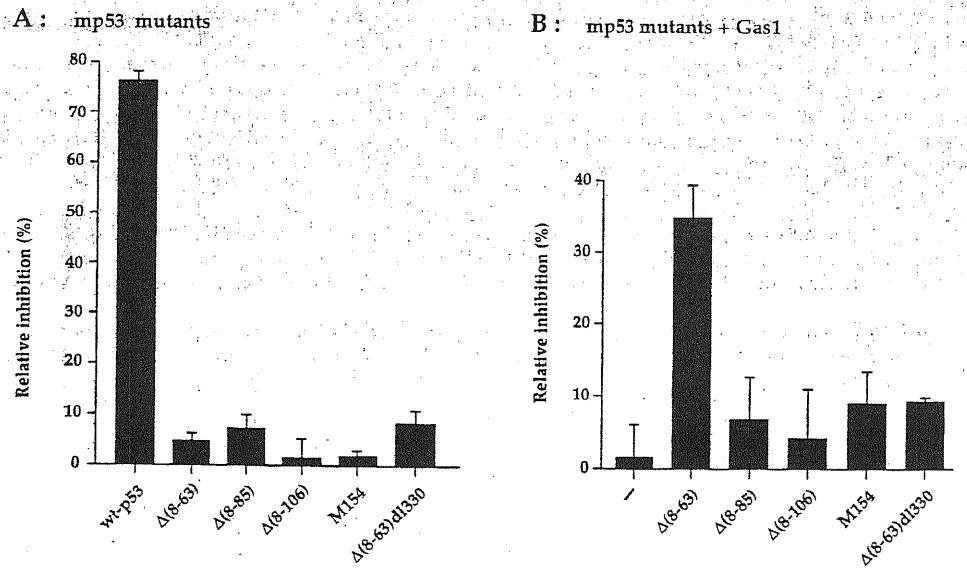

FIG. 2. Growth arrest analysis of the p53 deletion mutants in BALB/c (10)1 fibroblasts. (*A*) Relative inhibition of BrdUrd incorporation by expression of the various p53 mutants. (*B*) Relative inhibition of BrdUrd incorporation after coexpression of the various p53 mutants in combination with Gas1. pGDVS7 expression vector (100 ng/μl of each) was microinjected into the nuclei of growing BALB/c (10)1 fibroblasts. When Gas1 was expressed together with the p53 deletion mutants, 50 ng/μl of each plasmid was used. Eighteen hours after injection 50 μM BrdUrd was added to the culture medium to monitor S phase. After 6 hr cells were fixed and processed for immunofluorescence as described in *Materials and Methods*. The percentage of relative inhibition of DNA synthesis in the injected cells was calculated by the following formula: % relative inhibition = [% BrdUrd-positive cells (uninjected) − % of BrdUrd-positive cells (p53/Gas1-positive)/% of BrdUrd-positive cells (uninjected)]. The mean of three independent experiments with at least 300 overexpressing cells scored is shown.

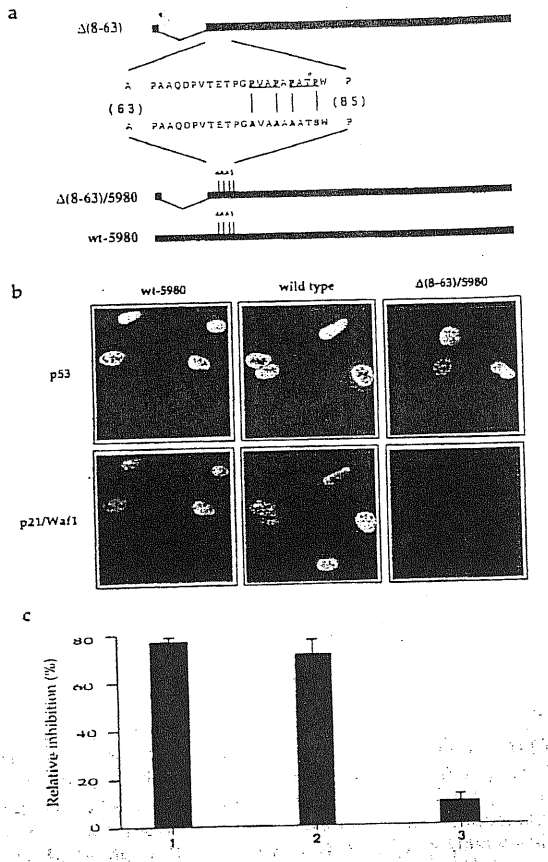

FIG. 3. Structure, p21/Waf1-induction and growth arrest function analysis of proline-rich region mutants. (*a*) Amino acid sequence of the proline-rich region in mouse p53. The PXXP motifs are underlined; the substituted amino acids in the mutated version are indicated in bold letters. Clone p53wt-5980 codes for a murine p53 bearing four point mutations that substitute Pro-76, -79, and -81 with alanines, and Pro-84 with serine. (*b*) Proline substitution in the p53 sequence does not interfere with its ability to transactivate endogenous p21/Waf1 as assayed by the induction of the corresponding protein product. Expression plasmids were microinjected in BALB/c (10)1 cells. Six hours later cells were fixed and analyzed for expression of the p53 protein and of endogenous p21/Waf1 by immunofluorescence. p21/Waf1 was detected by anti-peptide antibody as described (29). Wt p53 and Δ(8–63)/5980 were used as positive and negative controls, respectively. (*c*) Analysis of the growth arrest function of p53 wt-5980 (1), wt p53 (2), and Δ(8–3)/5980 (3). BrdUrd incorporation was monitored as described in legend to Fig. 2.

p53wt-5980 was comparable to that of wt p53, whereas Δ(8–63)/5980 was found to lack such activity (Fig. 3*c*).

Next, we analyzed the ability of the proline mutated Δ(8–63)/5980 to reconstitute the Gas1-dependent growth arrest function. Most remarkably, this mutant totally failed to cooperate with Gas1 to induce growth arrest, in stark contrast to the proline-containing Δ(8–63)mutant (Fig. 4). This strongly suggests that the proline-rich region defines a domain within p53 required to transduce a signal from the plasma membrane, where Gas1 is localized, eventually resulting in growth suppression.

We next wished to gain insight into the molecular mode of action of this domain. Given its evolutionary conservation, a reasonable hypothesis is that this domain may mediate growth suppression via Gas1-regulated interaction with a cellular protein containing an SH3 domain. We therefore reasoned that a truncated form of p53, retaining the proline-rich domain

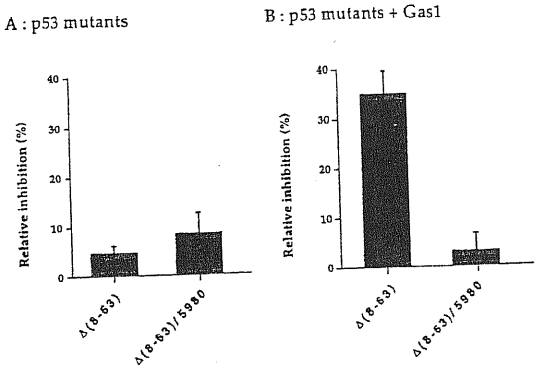

FIG. 4. Mutation of Pro-76, -79, -81, and -84 within deletion mutant p53 Δ(8–63) affects the ability to reconstitute the Gas1-dependent growth arrest function. (*A*) Relative inhibition of BrdUrd incorporation by expression of Δ(8–63) or Δ(8–63)/5980 p53 mutants. (*B*) Relative inhibition of BrdUrd incorporation by co-expression of the same p53 mutants in combination with Gas1. Microinjection experiments were performed as described in legend to Fig. 2. The mean of three independent experiments with at least 300 overexpressing cells scored are shown.

and the DNA binding domain but lacking both transactivation and oligomerization domains [Δ(8–63)dl330], might behave as an efficient competitor for the postulated interaction and prevent its biological consequences (Fig. 5). On the other hand such competition should not be exerted by the same truncated form of p53 missing the critical prolines [Δ(8–63)dl330/5980]. To test this prediction, we performed a triple co-expression experiment where either the Δ(8–63)dl330 or Δ(8–63)dl330/5980 truncation mutants were microinjected together with Gas1 and the Δ(8–63) mutant into BALB/c (10)1 cells. As seen in Fig. 6, the truncated Δ(8–63)dl330 mutant indeed interfered efficiently with growth arrest induced by the complementing co-expression of Gas1 and Δ(8–63), but this interference was abolished by mutation of the critical prolines, as in Δ(8–63)dl330/5980. Experiments were scored either for Gas1 or p53 expression relative to BrdUrd incorporation giving similar results. The "proline specificity" of the observed

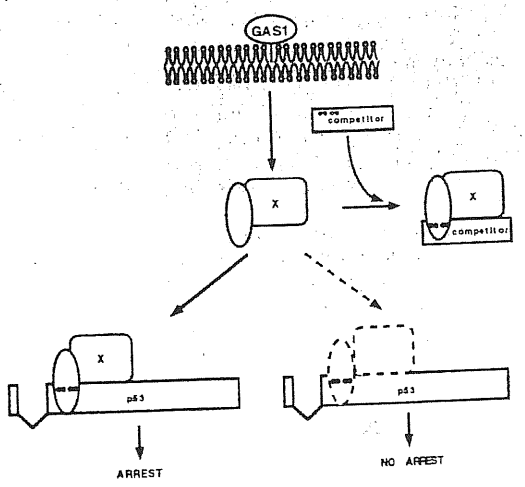

FIG. 5. Model for Gas1 signaling via the proline-rich domain of p53. The proline-rich domain of p53 (dots indicate the four relevant prolines) may signal for growth suppression via contacting the SH3 domain of a cellular protein (X). Such interaction should be modulated by signals departing from the plasma membrane where Gas1 is localized. A p53 mutant lacking the tetramerization domain (competitor) could interfere with growth suppression by sequestering the cognate SH3-containing cellular protein.

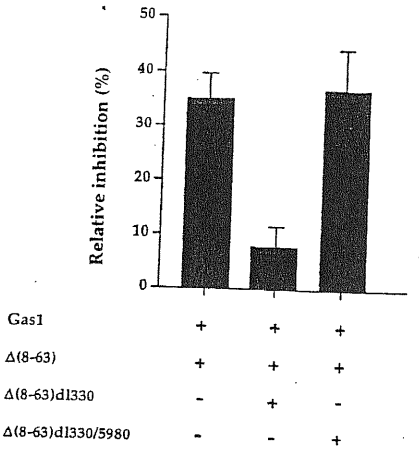

FIG. 6. Triple co-expression of Gas1 plus Δ(8–63) and either Δ(8–63)dl330 or Δ(8–63)dl330/5980 deletion mutants in BALB/c (10)1 cells. Δ(8–63)dl330 mutant, that retains the proline-rich domain but lacks the transactivation and oligomerization domains, behaves as an efficient competitor for the Gas1/Δ(8–63) inhibitory effect. Competition is not observed with the corresponding proline-substituted mutant Δ(8–63)dl330/5980. Microinjection experiments were performed as described in legend to Fig. 2. pGDSV7-gas1 (50 ng/μl) plus 30 ng/μl of pGDSV7-Δ(8–63), and 30 ng/μl of either pGDSV7-Δ(8–63)dl330 or pGDSV7-Δ(8–63)dl330/5980 were microinjected into BALB/c (10)1 cells. Experiments were scored either for Gas1 or p53 expression relative to BrdUrd incorporation giving similar results. Mean ± SD.

competition strongly supports the notion that a cellular protein(s) interacting with the proline-rich motif of p53 is responsible for mediating Gas1-induced growth arrest.

DISCUSSION

The p53 tumor suppressor protein is capable of responding to multiple cellular stress signals and, depending on the type of signal and the cellular context, the integrated p53 response can result in transient cell cycle arrest compatible with survival or apoptosis. A large body of evidence indicates the important function of p53 as a transcription factor, mediating such effects via the activation of a specific genetic program. Such activity, however, can sometimes be dispensable for the apoptotic p53 pathway (2). Therefore, given the complexity of stimuli that p53 is called to integrate into growth arrest/survival (42) or apoptosis, it is conceivable that, in addition to the transactivation function, different domains of the p53 protein directly modulate the transmission of specific signals by interaction with other cellular proteins. The availability of a biological assay that allows one to discriminate transactivation-independent p53 functions should therefore be instrumental in identifying such new domains and the mechanisms involved. In this context we have shown that ectopically expressed Gas1 exerts growth suppression activity in various cells types (27, 28) through a p53-mediated mechanism that is transactivation-independent (29). This represented a clear case where the transactivation function of p53 was dispensable for the p53-dependent biological response. We therefore wished to identify the region/domain of p53 that is specifically required for mediating the growth suppressing Gas1 signal. Analysis of various p53 deletion mutants established rigorously that the transactivation domain is structurally dispensable for Gas1 complementation in p53 null cells. Full DNA binding function of p53 was shown to be required but not sufficient for complementation. Mutants Δ(8–63) and Δ(8–85) were particularly instructive since only the former was able to complement Gas1. Both mutants retaining full DNA binding ability, the only difference between the two mutants is the presence of the proline-rich region contained between amino acids 63–85. Deletion of the corresponding region (amino acids 61–94) in human p53 impairs growth suppression of tumor cell growth in culture, as determined by colony formation assays (43). Such a proline-rich region therefore defines a new domain of p53, involved in the transactivation-independent transduction of the Gas1 growth arrest signal. Within this domain we were able to identify a critical motif, encompassing Pro-76–84 and representing a potential binding site for SH3 domains. Mutant Δ(8–63), containing substituted prolines at positions 76, 79, 81, and 84, was defective in complementing the Gas1-dependent growth arrest. Because of the well documented roles that PXXP motifs play in the formation of signaling complexes through the binding of cognate SH3 domains, we hypothesized that this motif could similarly act via the binding of a putative cellular protein involved in the transmission of the growth arrest signal. Our data suggest that, in addition to the PXXP motif, this interaction requires wt conformation of the nearby DNA binding domain. It is not uncommon that interactions between domains such as SH3 and docking sites are also influenced by the conformation of the surrounding domains (41). Interaction with a cellular protein, requiring both the PXXP and the DNA binding domain of p53, may represent a final regulatory event in the Gas1-dependent signal transduction cascade initiated at the plasma membrane (Fig. 5). The competition experiments support this working model. The truncated mutant containing the proline-rich domain and the DNA binding core was indeed able to block the complementation in the Gas1 growth arrest, but this was not observed using the proline mutant. A candidate protein, known to interact with p53 and containing an SH3 domain, could be 53BP2 (44, 45). The recently reported crystal structure of the complex between p53 and 53BP2 (46) demonstrates, however, that the SH3 domain of 53BP2 interacts with the L3 loop of p53's central core domain (amino acid241–248) in a manner distinct from that of previously characterized SH3 complexes. This leaves open the identity of the protein responsible for the interaction and the activities of the proline-rich domain described here. Altogether our results identify a novel functional domain within p53, which highlights the ability of p53 to directly participate in the transmission of antiproliferative signals in a manner not necessarily requiring its activity as a transcription factor. Such a domain likely represents a target site for interaction with elements of signal transduction pathways that transduce stress signals. In this scenario Gas1 might represent one element, located at the plasma membrane, that is involved in the transduction of a critical signal during G0 when Gas1 is maximally expressed. Identification of the cellular proteins capable of specifically interacting with the identified proline domain of p53 will greatly increase our understanding of this pathway and of p53 function in general.

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cDNA CHARACTERIZATION AND CHROMOSOME MAPPING OF THE HUMAN GAS2 GENE

As a secondary project, I have also been involved in the cloning, characterisation, and chromosomal mapping of the human homologue of the growth-arrest-specific gene GAS2.

Gas2 is a microfilament associated protein which is proteolytically processed by caspases during programmed cell death. Overexpression of the apoptotic form of Gas2 can induce dramatic cytoskeletal rearrangements in the cells [18].

A great deal of information about Gas2 was obtained by studying exclusively the murine protein, within murine cells. Therefore, it was important to clone and characterise the human Gas2 homologue, not only to extend the results to the human system, but also to provide informations and tools required for investigating a possible involvement of GAS2 in human disease. This work was further stimulated by the report that a human gene (GAR22) encoding a protein related to Gas2 was positionally cloned within a putative tumor suppressor locus at 22q12 [271].

SHORT COMMUNICATION

cDNA Characterization and Chromosome Mapping of the Human GAS2 Gene

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Murine Gas2 is a microfilament-associated protein whose expression is increased at growth arrest in mammalian cells. During apoptosis, Gas2 is specifically cleaved at its C-terminus by a still unknown ICE-like protease, and the processed protein induces dramatic rearrangements in the cytoskeleton when overexpressed in several cell types. Here we report the characterization of a cDNA encoding the human homologue of Gas2, showing high conservation with the murine counterpart at the protein level. Fluorescence *in situ* hybridization analysis and radiation hybrid mapping localized the GAS2 gene on human chromosome 11p14.3–p15.2, in a region homologous to the gas2 region on mouse chromosome 7. © 1998 Academic Press

The gas2 gene was isolated as a member of a set of genes specifically expressed at growth arrest in murine fibroblasts (3, 19). Accordingly, gas2 mRNA is downregulated upon serum stimulation of quiescent cells. Gas2 is a cytoplasmic protein colocalizing with the microfilament system, and upon growth-factor stimulation it becomes hyperphosphorylated and accumulates at membrane ruffles (5). An important posttranslational modification of Gas2 protein is observed during apoptosis, when Gas2 is processed by proteases of the caspase family (2). Increasing evidence points to a central role of caspases—ICE-like proteases (1)—in the process of programmed cell death, and it is believed that posttranslational activation of caspases, with the consequent modification of multiple cellular substrates, contributes to the profound cellular changes observed in apoptosis (9, 15, 16). In apoptotic cells Gas2 is specifically processed at a characteristic aspartic residue in the C-terminal domain, and overexpression of the apoptotic form of Gas2 induces dramatic rearrangements of the actin cytoskeleton (2). Altogether these data strongly suggest the involvement of Gas2 in the regulation of microfilament dynamics, during both cell cycle and apoptosis. Recently, a gene (GAR22) whose product is significantly similar to Gas2 has been cloned as a candidate tumor suppressor on chromosome 22q12, a region frequently involved in allelic losses associated with human cancer (22).

To clone the human homologue of murine gas2, a fragment of the murine cDNA was used as a probe to screen a human kidney cDNA library. The longest isolated clone was truncated at the 5′ end, missing most of the coding region. Screening of the dbEST database revealed two sequences (GenBank Accession Nos. R15728 and H82041) matching the 5′ region of the murine cDNA. These ESTs corresponded to IMAGE Consortium clones 66454 and 239955, both derived from a normal human fetal liver/spleen library (12). The structure and sequence of the two clones were analyzed in relation to the incomplete cDNA from kidney. Clone 66454 missed the 3′ end and was not analyzed any further. Clone 239955, 2 kb in length, corresponded to the clone from human kidney, extending it to the 5′ end, and will be hereon referred to as human gas2 (hgas2) cDNA. The sequence of hgas2 cDNA has been deposited with the GenBank/EMBL/DDBJ databases under Accession No. U95032.

Figure 1 shows the alignment of the murine and human Gas2 proteins as deduced from the respective cDNA sequences. In the human cDNA only the second of two adjacent in-frame ATG codons that are found in murine Gas2 appears to be conserved, and consequently the deduced protein is 1 amino acid shorter. The amino acid sequence is extremely conserved: it differs from the murine counterpart in only 8 residues, 7 of which represent conservative substitutions.

To analyze the intracellular localization of human

Sequence data from this article have been deposited with the EMBL/GenBank/DDBJ Data Libraries under Accession No. U95032.

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m   MMCTALSPKVRSGPGLSDMHQYSQWLASRHEANLLPMKEDLALWLTNLLGKEITAETF 58
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
h    MCTALSPKVRSGPGLSDMHQYSQWLASRHEANLLPMKEDLALWLTNLLGKEITAETF 57

m   MEKLDNGALLCQLAATVQEKFKESMDANKPAKTLPLKKIPCKASAPSGSFFARDNTAN 116
    |||||||||||||| | |||||||||||| | ||||||||| ||||||||||||||||
h   MEKLDNGALLCQLAETMQEKFKESMDANKPTKNLPLKKIPCKTSAPSGSFFARDNTAN 115

m   FLSWCRDLGVDETCLFESEGLVLHKQPREVCLCLLELGRIAARYGVEPPGLIKLEKEI 174
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
h   FLSWCRDLGVDETCLFESEGLVLHKQPREVCLCLLELGRIAARYGVEPPGLIKLEKEI 173

m   EQEETLSAPSPSPSPSSKSSGKKSTGNLLDDAVKRISEDPPCKCPTKFCVERLSQGRY 232
    |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
h   EQEETLSAPSPSPSPSSKSSGKKSTGNLLDDAVKRISEDPPCKCPNKFCVERLSQGRY 231

                                                  *
m   RVGEKILFIRMLHNKHVMVRVGGGWETFAGYLLKHDPCRMLQISRVDGKTSPVQSKSP 290
    |||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
h   RVGEKILFIRMLHNKHVMVRVGGGWETFAGYLLKHDPCRMLQISRVDGKTSPIQSKSP 289

m   TLKDMNPDNYLVVSATYKAKKEIK 314
    |||||||||||||| ||||||||
h   TLKDMNPDNYLVVSASYKAKKEIK 313
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FIG. 1. Alignment of the deduced peptides corresponding to human (h) and mouse (m) Gas2 proteins. The asterisk indicates the Asp residue recognized by caspases, defining the C-terminal domain that is cleaved during apoptosis.

Gas2, the insert from clone 239955 was subcloned in the pGDSV7 expression vector (2) and transfected in COS7 cells. As shown in Fig. 2, the human protein, detected by a polyclonal antibody against murine Gas2, is localized at the actin cytoskeleton, along the stress fibers and at the plasma membrane (Fig. 2A), as confirmed by comparison with the distribution of polymeric actin (Fig. 2B).

Proteolytic cleavage of murine Gas2 can be readily detected in apoptotic cells. To confirm that the human protein is analogously processed, the construct pGDSV7-hgas2 was transfected in COS7 cells that were subsequently cultured for 48 h in serum-free medium. Under these conditions apoptotic cells detach from the petri dish and can be recovered in the culture medium for biochemical analysis (2). Figure 2C reports the Western blot analysis of lysates from adherent (live) versus nonadherent (apoptotic) transfected COS7 cells. Human Gas2 is cleaved in apoptotic cells, as assessed by the increased mobility of the protein (lane 2), and the processed protein is not recognized by an antibody specific for the C-terminal domain of murine Gas2 (lane 4), thus confirming that apoptotic cleavage removes the C-terminal part of the protein.

Expression of Gas2 in human tissues was evaluated by Western blotting and RT-PCR experiments on autoptic samples. The human GAS2 transcript could be detected by PCR in most of the tissues analyzed (not shown), while Western blot analysis indicated that Gas2 protein was expressed at highest levels in liver, lung, and kidney (see Fig. 3), in agreement with the expression pattern observed in murine tissues (19) (C.B., unpublished data). We can therefore conclude that human Gas2 shares both structural and functional homology to murine Gas2, with a similar pattern of tissue distribution, similar intracellular localization, and analogous proteolytic processing during apoptosis.

To map the human GAS2 gene on human chromosomes, the entire hgas2 cDNA was used to screen a genomic library from human placenta. Two separate lambda clones were selected from several positive signals. One clone (λ-4.2) contained a single exon corresponding to nt 545–687 of hgas2 cDNA; the other (λ-3.1) contained a downstream exon starting from nt 796. Neither of the two clones contained the intervening sequences corresponding to nt 688–795 of the cDNA, and therefore they represented nonoverlapping segments of the human GAS2 gene. Chromosomal localization was performed by *in situ* hybridization on metaphase chromosomes using the nonisotopic technique based on the biotin–avidin detection system. Biotin-labeled phage clones λ-4.2 and λ-3.1 were hybridized,

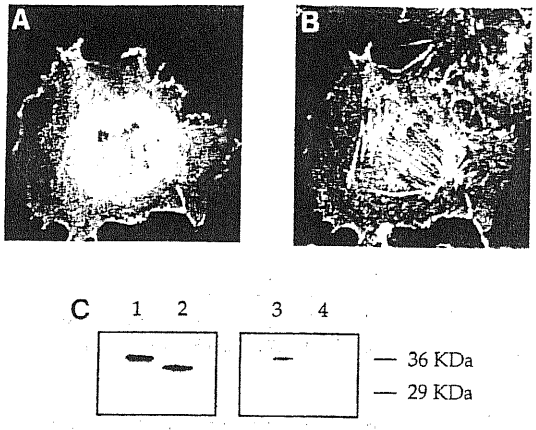

FIG. 2. (A and B) Intracellular localization of human Gas2 protein in transfected COS7 cells. (A) Gas2 was detected by a polyclonal antibody against murine Gas2 followed by a FITC-conjugated anti-rabbit secondary antibody. (B) Cellular actin was stained with rhodaminated phalloidin. Cells were examined with a Zeiss laser scan confocal microscope. (C) Western blot analysis of transfected human Gas2 in live adherent (lanes 1 and 3) versus apoptotic nonadherent (lanes 2 and 4) serum-starved COS7 cells. Gas2 was detected by two different polyclonal antibodies: one raised against the entire Gas2 protein (lanes 1 and 2), the other raised against a C-terminal peptide (lanes 3 and 4). The experiment was performed as described in (2).

both separately and together, to human metaphase chromosome spreads obtained from normal males and females. *In situ* hybridization, detection with FITC-conjugated avidin, and chromosome G-banding were performed as previously described (18). In 28 metaphase cells examined, a total of 130 fluorescent signals were scored on chromosomes. Of these, 93 (72%) were clustered in the 11p14–p15 region, while the others were randomly distributed on other chromosomal sites. Of the dots recorded on chromosome 11, a peak of 63 fluorescent signals (68%) was localized on band p14.3–p15.2 (Fig. 4G). These data, obtained by analyzing mainly prometaphase chromosomes (Figs. 4C, 4D, 4E, and 4F), enabled us to assign GAS2 to the p14.3–p15.2 region of human chromosome 11.

The GAS2 chromosomal localization was confirmed by radiation hybrid (RH) mapping using the GeneBridge 4 panel (provided by the Human Genome Mapping Project Resource Centre, UK), which includes 93

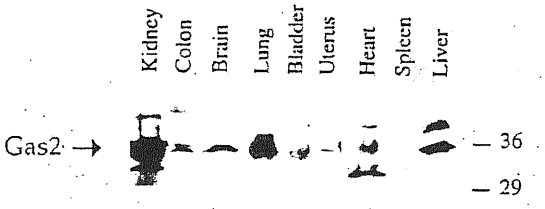

FIG. 3. Expression of Gas2 in human tissues. Autoptic samples from the indicated tissues were analyzed for expression of human Gas2 by immunoblotting. The position of Gas2 protein is indicated. Western blots were performed as previously described (2), using a polyclonal antibody raised against the entire murine protein.

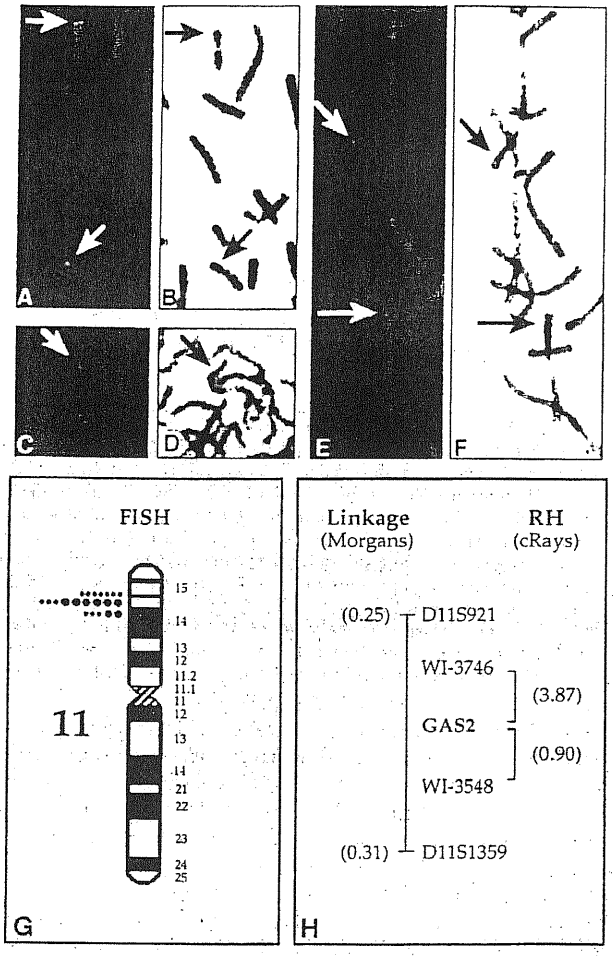

FIG. 4. Localization of the human GAS2 gene to chromosome 11p14.3–p15.2. Human metaphase (A) and prometaphase (C and E) chromosomes were hybridized with biotin-labeled phage clones λ-4.2 and λ-3.1. Hybridization was detected by FITC-conjugated avidin, and chromosomes were stained with propidium iodide. (A, C, and E) Partial metaphases showing hybridization signals on chromosome 11. (B, D, and F) Partial metaphases shown in A, C, and E, respectively, G-banded with Wright's stain. (G) Idiogram of G-banded human chromosome 11 showing the distribution of the hybridization signals (each large dot represents 10 fluorescent signals). (H) Schematic table indicating the GAS2 position as determined by RH mapping.

human/hamster clones (21). PCR amplification was performed on the DNA of somatic cell hybrids using two primers, GENGAS2UP (5′-ACACTAATTTCACTAGAACCAG-3′) and GENGAS2LO (5′-AGAGACCACCAAGTAGTTATCT-3′), the former designed in the intron and the latter in the exon of the genomic sequences of clone λ-3.1, to generate a 272-bp specific product that could not be detected in the hamster controls. Data were submitted to the RH mapping server at Whitehead Institute/MIT Center for Genome Research, which provides an MIT framework marker linked to the subject STS with a lod score >3.0 (8). The GAS2-

specific PCR-amplified product was mapped within band 11p15.2, 3.87 cR proximal to WI-3746 and 0.9 cR distal to WI-3548 (see Fig. 4H). The chromosomal localization indicates that the human GAS2 gene is syntenic to the murine GAS2 gene, which was previously localized on mouse chromosomal band 7C (6), a region syntenic and compositionally related to the distal short arm of human chromosome 11 (7, 17).

Rearrangements or deletions of the short arm of chromosome 11 are frequently found in human tumors (14). Recently, several authors have reviewed the many evidences linking loss of heterozygosity at the distal part of chromosome 11p with a variety of sporadic human cancers (11, 13, 20): the existence of two putative tumor suppressor loci within the 11p15 region was further demonstrated using subchromosomal transferable fragments (10, 11), and identifying mutations of genes in this region is an effort of many laboratories. The mapping of human GAS2 on 11p14.3–p15 adds another interesting candidate to the list of genes that lie in this chromosomal region (10, 13). Gas2 is a component of the microfilament system, its expression is increased at growth arrest, and its intracellular localization is tightly regulated upon serum stimulation of quiescent cells. During apoptosis, Gas2 is specifically cleaved by caspases at its carboxy terminus, and removal of the Gas2 carboxy-terminal domain unveils a potent microfilament and cell shape reorganizing activity (2). This feature makes Gas2 a good candidate apoptotic effector, regulating the specific changes of the microfilament system that occur during apoptosis. Recently, it has been demonstrated that Gas2 expression correlates with increased susceptibility to apoptosis. Specifically, the increased Gas2 expression observed after serum starvation was shown to be unrelated to growth arrest but instead coupled to a massive apoptotic response (4). This suggests that Gas2 might play a double role in apoptosis, as an effector of the apoptotic program, under direct control of a caspase, or as a modulator of the apoptotic program itself, regulating the susceptibility to apoptosis in response to specific signals.

As shown here for the human protein, expression of Gas2 in tissues appears not to be ubiquitous (3, 19), arguing against the hypothesis that Gas2 is a universal effector of microfilament transitions during apoptosis, but again suggesting a function of this gene in modulating susceptibility to apoptosis in response to specific environmental stimuli. Other effectors functionally and structurally related to Gas2 might therefore exist. Interestingly, the protein encoded by GAR22, a candidate tumor suppressor gene on chromosome 22q12, shows 35% identity to both human and murine Gas2 proteins (22); this indicates that Gas2 could be member of a gene family. Given the mentioned biological characteristics of Gas2 and its involvement in important cellular processes such as growth arrest and apoptosis, definition of its chromosomal localization opens the way for analysis of potential alterations of this gene in human disease.

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CONCLUSIONS

A very strong scientific effort during the last 19 years has provided a massive amount of information about p53 function. It has been found that p53 can interact with a large number of cellular and viral proteins, it can transactivate a long list of cellular genes, it represses transcription from a number of cellular and viral promoters, and even performs peculiar biochemical activities such as 3'-5' exonuclease, or re-annealing of single stranded RNA and DNA (see PART1).

p53 is mutated or deleted in more than half of human cancers [95], and in a number of tumors where the gene is not mutated it is functionally inactivated by viral or cellular oncogenic proteins. p53 plays a fundamental role in preserving genomic stability, so that cells lacking functional p53 are more prone to mutation and more likely to gain advantage within the highly selective environment of a growing tumor [126, 133]. Genetic stability is also influenced by other parameters in addition to DNA damage repair, for instance the accuracy of chromosome segregation, and the control of cellular ploidy. Accordingly, a number of evidences suggest that p53 plays an important function in monitoring the centrosome cycle [73], and in interfacing the spindle-assembly checkpoint with the control of DNA replication [36, 128]. Furthermore, a role for p53 in sensing alterations of cellular metabolism is also conceivable, since p53 is activated by inhibitors of ribonucleotide neosynthesis, as well as by heat/cold shock [138, 182], and p53 might indirectly regulate angiogenesis, cytoskeleton dynamics, growth-factor signalling (see PART1), and even GTP levels [217].

The significance of studying p53-regulated genes

The key role of p53 in development of human cancer makes it an important and very appealing subject of study, in view of finding therapeutic approaches and diagnostic markers for human tumors. What makes p53 unique in its importance, is the complex integration that it operates between detection (and evaluation) of cellular stress and

regulation of basic processes such as cell-cycle progression, apoptosis, and eventually DNA repair and differentiation. This means that studying p53 function might provide insight into the cellular mechanisms regulating such processes. In other words, effector genes regulated by p53 should be themselves components of regulatory pathways modulating such processes, even independently of p53 activation. A striking example is p21Waf1, which turned out to be a constitutive subunit of CDK complexes and a universal inhibitor of their activity, playing multiple roles, being normally regulated by p53-independent pathways [57, 94, 149].

Considering the list of accredited and putative p53-inducible genes sketchily described in PART 1, I think it emerges that probably there are no master effectors mediating selected p53 functions but rather, diverse patterns of genes are differentially regulated by p53, in response to different stimuli, to elicit different responses. Therefore, to fully understand p53 biological activities it would be necessary to obtain a complete picture of the gene expression patterns associated to each specific phenotype. In parallel, a detailed study of single effector genes is necessary to give functional significance to the expression data and offers the opportunity to characterise key molecular components of pathways regulating cell-proliferation and cell-death.

Regarding the possibility to analyse complex patterns of gene expression, the Genome and EST sequencing projects are slowly but inexorably building up a data base which will eventually contain all human genes; while the concomitant development of technologies such as cDNA micro-arrays and DNA-chips is providing the tools for literally taking "snapshots" of gene expression profiles. As discussed in PART 1, data are already beginning to be produced through the first pioneering applications of such techniques to the study of p53-dependent gene expression. Examples are the SAGE profile of genes regulated during p53-dependent apoptosis in human colon-cancer cell lines, published by the Vogelstein group [197], or the cDNA array-based analysis of the genes regulated *in vivo* in different tissues of mice exposed to whole body ionising radiation, recently published by Gudkov and co-workers [123].

But in the end, these novel approaches, as well as the "old fashioned" techniques of subtractive hybridisation and differential display, invariably produce a list (be it shorter or longer) of genes whose expression is induced or repressed upon p53 activation. Some of them might be known genes, some of them might be novel, some of them might be novel variants of known genes. The really challenging issue is defining the functional role of such genes in the context of p53-related activities.

Considerations on the results and future objectives

The work presented in this Thesis stems from a project devised to analyse the pattern of gene expression induced by wt p53 in the context of a reversible growth-arrest phenotype. The experimental system consisted of a murine cell line stably expressing a temperature-sensitive p53 allele. These cells promptly respond to conformational activation of p53 by arresting in G1 (and G2). Such arrested state in not associated to cell-death, and is essentially reversible; when p53 is switched back to mutant conformation these cells re-enter the cell-cycle and start proliferating again. The genes regulated by p53 in these cells, through their functional interactions with cellular products, are therefore candidate effectors of a "reversible cell-cycle arrest" function of wt p53. It is likely that such phenotype involves two principal categories of gene products: some involved in braking the cell-cycle, directly contributing to growth-arrest; other involved in dynamic maintenance and regulation of the growth-arrested status.

Six p53-inducible transcripts were isolated in this experimental system [238].

I focused my research activity on the characterisation of two of these clones, with the scope of delineating their possible functional role in the context of p53 activities.

B99. A novel microtubule localised protein which might be involved in G2-specific functions of wt p53

The B99 gene, described in PART 2, encodes a novel protein localised to the microtubule network. B99 mRNA and protein are induced by wt p53 in diverse experimental conditions, and the B99 gene contains a functional p53-responsive element. Interestingly, I found that expression of endogenous B99 protein upon p53 activation is restricted to the cell population with 4N DNA content (G2/M fraction). I presented evidence that B99 is cell-cycle regulated even in the absence of DNA damage or other p53 stabilising stimuli, displaying a cyclin-like behaviour, and I also presented some observations suggesting that B99 protein might be subject to complex post-translational regulation. Notably, when B99 was overexpressed in murine or human fibroblasts it inhibited cell growth and caused accumulation of cells with 4N DNA content, indicative of interference with G2/M progression. Altogether, these results point to B99 as an appealing candidate mediator of specific biological activities of wt p53 during late S/G2 phase.

It is evident that B99 presents a number of interesting features. First of all it is a novel gene, encoding a protein with no strong homology to any protein deposited in databases. B99 is efficiently regulated by p53 both at the mRNA and protein levels, it promptly responds to DNA damage, and contains a functional p53-RE in its genomic sequences. Therefore, I think that B99 meets the most stringent definition of a p53-inducible gene and this is very important, since it confirms the validity of our model system and the reliability of our screening approach. Second, B99 combines properties such as G2-specificity of expression, microtubular localisation and induction of a G2-delay, with an efficient p53-responsiveness.

Regarding B99 expression, p53-dependent induction of the B99 protein appears to be restricted to late S/G2 phase. This observation suggests that either the p53-RE in B99 can only be recognised by a "G2-specific" p53 (e.g. phosphorylated by G2-specific CDKs), or the B99 promoter is responsive to stimulation by p53 only during late S/G2 phase. This last hypothesis implies that cell-cycle dependent regulation overlaps with p53-dependent regulation, blocking transcriptional activation by p53 in conditions in which the basal promoter would be off (e.g. during G0/G1). In this regard it would be interesting if B99 was subject to a regulation similar to other S/G2 specific genes such as cycA and cycB, cdc25c or Plk, which are transcriptionally repressed during G0/G1 by a specific factor (CDF1) [140, 272].

Regarding B99 microtubular localisation, it has a number of possible implications. For instance, if B99 directly interacts with microtubules, it might regulate microtubular rearrangements, thus acting as a marker of "p53-arrested cell"-specific cytoskeletal dynamics. It is worth remembering that wt p53 transcriptionally downregulates MAP4 and many tubulin isoforms, so that the microtubule composition of a p53-arrested cell might indeed be quite different from that of a normally proliferating cell [175]. It will therefore be important to determine whether B99 is a MAP, and can affect to any extent the dynamics of microtubules. Additionally, B99 might be involved in the suggested checkpoint functions that link p53 to microtubules. Loss of p53 has been clearly associated to development of abnormal number of centrosomes in murine cells [73]. Centrosomes mediate structural organisation of the microtubule network during interphase, and they are crucial during mitosis, since they direct assembly and polarity of the mitotic spindle. A tight regulation on the centrosome replication cycle is essential for assembly of a normal spindle and accurate segregation of the chromosomes; thus, an abnormal centrosome number strongly favours chromosome instability and aneuploidy. Given that B99 is localised to microtubules and can also stain the centrosome (see Figure 13a), it could be hypothesised that B99 might have a role in linking p53 function to the

centrosome cycle. To test this hypothesis we have just started a collaboration with dr. Levine to investigate if B99 can recover the centrosome defect in p53-null murine cells.

A number of evidences indicate that p53 can sense the status of microtubules and eventually be activated in the presence of microtubule poisons [117, 168, 235]. Furthermore, it has been shown that in the presence of spindle inhibitors, murine cells which cannot complete mitosis accumulate in pseudo-metaphase, but after some time they adapt, decondensing chromosomes, and returning to a state biochemically similar to G1 but having a 4N DNA content [128]. p53 plays a crucial role in preventing such adapted cells from undergoing a second round of DNA synthesis [36, 128]. This regulation has very important implications since loss of this checkpoint brings to polyploidy, a landmark of late stages in cellular transformation. Although there are evidences that Waf1 and the Rb pathway are involved in this checkpoint [117, 128], it is still absolutely obscure how p53 senses microtubule disruption, or the adapted, pseudo G1 condition [128]. Some preliminary experiments indicate that although cyclin B is downregulated, B99 protein is still expressed at significant levels in nocodazole adapted NIH3T3 cells (data not shown), thus distinguishing them form G1 cells. It would be therefore tempting to speculate that B99 plays some role in marking the 4N-DNA condition, and is somehow involved in p53-dependent aspects of the spindle checkpoint.

A correlation between microtubules and apoptosis has also been proposed, and microtubule-active drugs appear to influence the phosphorylation state of Bcl-2, thereby modulating cellular sensitivity to pro-apoptotic stimuli [88]. The microtubule-stabilising protein MAP4 is directly downregulated by p53 during p53-induced apoptosis [175], and MAP4 can influence p53-dependent responses to microtubule-poisons [265]. Since B99 has been cloned in a cellular system where p53 activation induces growth-arrest without apoptosis, it would be interesting to test whether B99 levels influence cellular sensitivity to apoptotic stimuli.

Regarding the observed effect of B99 in causing G2 delay, it is possible that B99 is a negative regulator of G2/M transition, selectively induced when p53 is activated in cells

that have traversed the G1/S boundary. I showed that B99 has indeed some growth-suppressive properties and causes an increase in the 4N fraction of the cell population, indicative of a longer time lapse between S phase and mitosis. It is possible that G2 delay comes from inhibition of the G2/M transition; this could be due either to a regulatory effect (i.e. B99 delays activation of MPF) or to a "mechanical" effect (i.e. B99 stabilises microtubules, hindering mitotic rearrangements). But it is also possible that the observed accumulation of 4N DNA population reflects a delayed exit from late stages of S phase, with cells bearing a quasi-4N DNA content essentially indistinguishable from *bona fide* G2 phase. Some observations might apparently argue with the hypothesis that B99 efficiently blocks G2/M transition: in fact cells overexpressing B99 can still incorporate BrdU (data not shown) and can still enter mitosis (although with lower efficiency). It cannot be excluded that B99 normally functions in concert with other p53 effectors (e.g. p21Waf1 inhibiting B/cdc2, and 14-3-3s inhibiting cdc25c) to block G2/M transition in cells undergoing p53-dependent G2 arrest.

AK1β. A membrane associated form of Adenylate Kinase affecting the kinetic performance of p53-dependent growth-arrest

One of the six clones isolated during the differential screening in Val5 contained only the 3' UTR region of murine cytosolic Adenylate Kinase (AK1), a highly conserved monomeric enzyme involved in cellular homeostasis of Adenine nucleotides. The corresponding probe detected a mRNA which was strongly induced by activation of ts p53, and was accumulated upon UV-treatment in a p53-dependent manner. Further characterisation revealed that such transcript encoded an alternative form of Adenylate Kinase, which I named AK1β, and which differs from AK1 for the presence of 18 extra aminoacids at the N-terminus. These extra residues provide a consensus signal for N-myristoylation and I showed that AK1β is localised to the plasmamembrane.

Interestingly, the human AK1 gene contains a number of putative p53 binding sites and I presented evidence that a similar regulation and induction of the alternative AK transcript is conserved in human cells. Finally, I described antisense ablation experiments suggesting that AK1β might play a critical role in establishing the reversible cell-cycle arrest induced by p53 in Val5 cells.

The above summarised work has two principal implications. First of all, I experimentally observed a p53-dependent regulation which had been previously hypothesised exclusively on the basis of a computer analysis for consensus p53-binding sites [17]. Even if I did not demonstrate that such putative p53-BS can be functional in reporter assays or gel mobility shifts (EMSA) *in vitro*, I provided indirect evidence that the gene is indeed p53-regulated *in cells*. This result conceptually supports the validity of the computer-based approach. Nonetheless, I found that p53 specifically induces a novel, alternative form of AK1, which could have never been predicted through computer-based sequence analysis. Therefore, even with the definition of a stringent and very reliable consensus, the presence of such a sequence within a gene might be strongly indicative, but not sufficient to predict that p53 will induce expression of the gene. This also implies that when we use high throughput expression screenings such as those based on SAGE or cDNA microarrays, we should be aware that such techniques may not detect small differences in the transcripts if they have not been described before. Very hardly a SAGE tag, or a cDNA probe could have distinguished AK1 from AK1β.

Second, I found that wt p53 recruits a protein normally involved in basal metabolic processes and enrolls it for some activity related to the establishment of growth-arrest. In fact, expression of an antisense mRNA directed to the common 3'UTR region of AK1 and AK1β induces a delay in p53-dependent growth-arrest of Val5 cells (see figures 6 and 7 in PART 3). I provided significant evidence that the effect of the antisense is due to interference with AK1β induction, suggesting that AK1β might somehow "help" in establishing cell-cycle arrest. It is important to point out that overexpression of AK1β

alone had no effect on the cell-cycle of growing, unstressed cells (not shown), and apparently did not enhance p53-dependent growth arrest, at least under the experimental conditions used in this study. Therefore, if AK1β plays some function, this function is probably co-operative and correlated with the cellular context. It would be tempting to speculate, for instance, that membrane localised AK1β is damping some signalling pathways that act as accelerators on the cell-cycle engine (i.e. growth-factors) thus making the job of cell-cycle brakes (i.e. p21Waf1) easier. This hypothesis would conceptually imply that p53-dependent regulation of the CDKs-pRb pathway is only one aspect of its growth-suppressive function; at least under certain circumstances, p53 might stimulate other regulators which cut upstream the signalling of growth-promoting (or survival) pathways. In line with this hypothesis, it has been proposed that p53 induces expression of RGS14, a negative regulator of G-protein coupled mitogenic signalling [23]. Within the frame of a growth-signal-damping hypothesis, AK1β might facilitate the kinetic establishment of growth-arrest upon activation of wt p53 or might contribute to maintain a long term cellular arrest as observed in Val5. Previous studies have shown that the kinetics of cell-cycle re-entry of p53-arrested Val5 cells after temperature shift up are influenced by the time of permanence in the G1 arrested status. The longer Val5 are kept at 32°C, the longer they take to enter S-phase after shifting to 37°C ([44] and G. Del Sal, unpublished results). It would be therefore interesting to evaluate if a correlation exists between the levels of AK1β accumulated in Val5 cells and the time required to re-enter in cycle after p53 inactivation. If this correlation indeed exists, this working hypothesis whould be tested in other cellular systems and more physiological p53-dependent responses.

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The work presented in this Thesis stemmed from a screening for p53-induced genes which has been subject of the PhD thesis of dr. Renè Utrera. I joined him for the final "easy" screening and harvesting of the clones, but he had previously spent more than two years of very hard work in developing the subtractive hybridization protocol that gave us these genes. I wish to acknowledge his steady effort and determination.

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