p53 CONTROL OVER CELL CYCLE PROGRESSION AT G2

by

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

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Abstract

Tp53 (the human form) is the most commonly mutated tumour suppressor gene found in a large variety of cancers, and appears to be involved in several functions, including DNA damage response, related suppression of inappropriate cell growth, and apoptosis. Wild type p53 is capable of inducing cell cycle arrest at specific points in the cell cycle, an ability lost by most p53 mutants. The first block occurs at the Restriction point before commitment to DNA replication (S). In this thesis, I examined whether p53 can also induce cell cycle arrest at the G2/M boundary of the cell cycle, and by what mechanism this is accomplished . To study this I used the REF52 cell line and the temperature sensitive p53 mutant, p53val^{135}. Cells were enriched in S phase before the temperature shift. REF52 cells expressing mutant p53val135 alone or with activated H-ras gene arrest primarily at the G1/S and G2/M parts of the cell cycle at the restrictive temperature, as determined by flow cytometric analysis. This suggested that the antiproliferative activity of p53 may be involved in regulation of the cell cycle at the G2/M restriction point as well as transit through G1/S and initiation of DNA synthesis.

I further demonstrated that Cdc2 kinase was inactive in p53 induced G2 arrested cells. G2 arrest appears to operate through a mechanism controlling the phosphorylation state of Cdc2. Cells arrested by p53 maintain phosphorylation of the regulatory tyrosine 15 amino acid on Cdc2 kinase. The levels of Cdc2 protein as well as the relevant cyclin, B1 were unaffected. Addition of caffeine, a known checkpoint inhibitor, both prior to and after establishment of G2 arrest can overcome this event, but not the G1 arrest. Examination of one Cdc2 kinase regulatory phosphatase, Cdc25C, showed the protein hypophosphorylated

in p53 induced G2 REF52 arrested cells. This protein became phosphorylated upon addition and release by caffeine in these cells. From this I conclude the p53 induces arrest at G2 by regulation of phosphorylation and activation of Cdc2 kinase, and completion of the cell cycle.

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List of Abbreviations

ara-C	Antimetabolite 1-β-D-arabinofuranosylcytosine
ATP	Adenosine triphosphate
bp	Base pair
BPDE	*anti*-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene
BRK	Baby rat kidney
CKI,II	Casein kinase I, II
Cdk	Cyclin dependent kinase
cpm	Counts per minute
Ci	Curies(s)
°C	Degrees celcius
CBP	CREB-binding protein
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
FCS	Fetal calf serum
G2	Gap2
GML	GPI-anchored molecule like protein
GPI	Glycosyl-phosphatidylinositol
HPV	Human papilloma virus
HU	Hydroxyurea
ICE	Interleukin 1 β converting enzyme

Ig	Immunoglobin
IGF-BP3	Insulin-like growth factor binding protein
Kd	Kilodalton
MAPK	Mitogen activating protein kinase
MMS	Methyl methanesulfonate
µg, mg, g	Microgram, milligram, gram
µl, ml, l	Microlitre, millilitre, litre
µM, mM, M	Micromolar, millimolar, molar
Mdm	Murine double minute
p53as	Splice variant form of p53
53BP1,2	P53 binding protein 1,2
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PKA,C	Protein kinase A,C
PP	Polyproline
PP2A	Protein phosphatase 2A
Rb	Retinoblastoma
RPA	Replication Protein A
rpm	Rotations per minute
SAP/JNK	Stress activated kinase
SV40	Simian virus 40
SDS	Sodium dodecyl sulfate

SS	Serum starved
TBP	TATA binding protein
TBS	Tris buffered saline
tet	Tetracyclin
TF	Transcription factor
TRIS	Tris(hydroxymethyl)aminomethane
wt	Wild type

Literature Review:

1 Introduction

The tumour suppressor gene p53 codes for a multifunctional protein which regulates numerous processes in the cell. P53 has been implicated in control of cell growth, differentiation, senescence, apoptosis, angiogenesis and DNA damage response (for review, see (Ko and Prives, 1996)). The role p53 has in regulating control over cell cycle progression during the G2 phase of the cell cycle is the subject of this thesis work. The following is a discussion of the general properties of p53, control over the cell cycle at G2 (Gap2), and how they relate to p53's control over cell cycle progression at G2.

1.1 Discovery of p53

P53 was first identified nearly 20 years ago, both as a SV40 large T antigen binding protein of 53 kD (Linzer and Levine, 1979; Lane and Crawford, 1979), and as a tumour antigen (DeLeo et al. 1979). Original investigations classified p53 as a oncogene, based on amongst other things it's ability to increase the life span in culture of rat chondrocytes (Jenkins et al. 1984), and it's ability to cooperate with oncogenes such as Ha-ras in the transformation of rat embryo fibroblasts (Eliyahu et al. 1984; Parada et al. 1984; Hinds et al. 1987). Subsequent investigation, however, demonstrated that this transforming potential was limited to mutant forms of p53 (Hinds et al. 1989), and that the wild type protein was in fact anti-oncogenic. Wild-type p53 when introduced into cells was found to be growth suppressive (Eliyahu et al. 1989; Finlay et al. 1989). Further analysis of both murine and

human tumours demonstrated that p53 was either mutated or entirely lost (Mowat et al, 1985; Baker et al. 1989; Nigro et al. 1989; Takahashi et al. 1989), and that p53 mutations occurred at a high frequency (Hollstein et al. 1991). It was also noted that inactivation of p53 was an important step during transformation of cells by DNA viruses such as SV40 (Linzer and Levine, 1979; Lane and Crawford, 1979), adenovirus (Sarnow et al. 1982; Zantema et al. 1985) and HPV 16 and 18 (Scheffner et al. 1990). Together this suggests that p53 occupies a central and important role in control over normal cell growth, one which must be overcome to permit transformation and carcinogenesis.

1.2 Structure of p53

P53 is a molecular "Jack of all trades", with many different activities. In humans, the gene for p53 resides on the short arm of chromosome 17 (17p13) (Isobe et al. 1986; Baker et al. 1989). The p53 gene produces two alternatively spliced products, encoding the p53 protein (major) 53 kd form, as well as a naturally occurring splice variant termed p53as. p53as is identical to the major form except for a small portion of the carboxy terminus (containing the negative regulatory region for DNA binding and the Pab421 antibody site) which is replaced with a short stretch of unique amino acids ((Han and Kulesz-Martin, 1992; Kulesz-Martin et al. 1994; Wu et al. 1994), see Figure 1).

The major protein is comprised of three functional domains (summarized in Figure 2). The first is the amino terminal transcriptional activation region (encompassing amino acids 1-43). This region also interacts with several members of the general transcription initiation complex, including the TATA box binding protein (Seto et al. 1992; Liu et al.

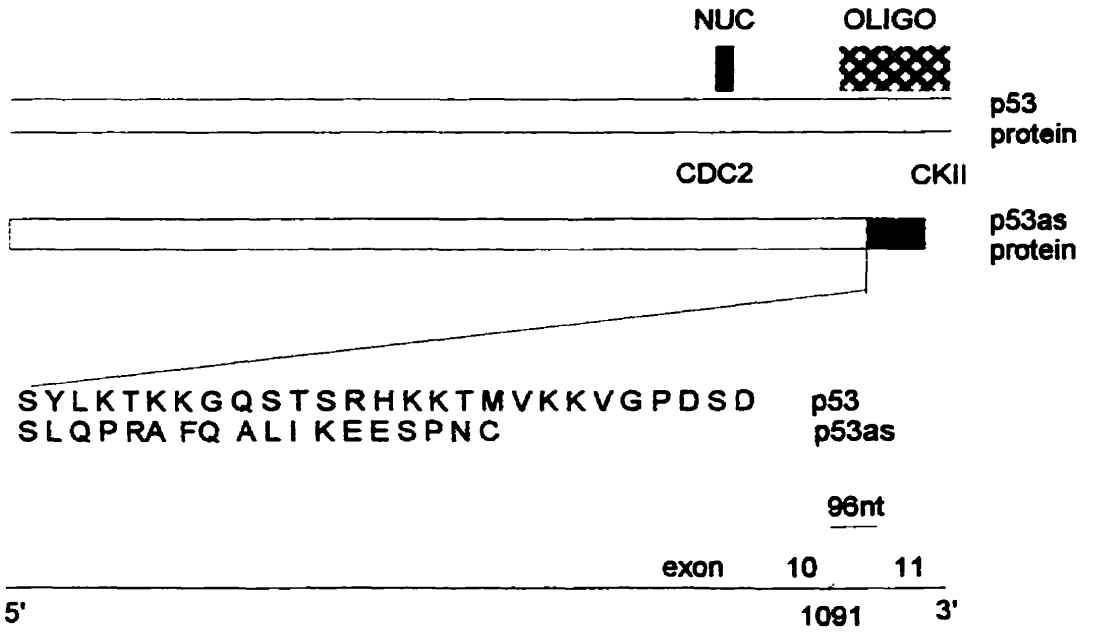

Figure 1: Protein products of the p53 gene. P53as protein is derived from an alternative splicing event occurring in exon 10. This event results in the loss of the carboxyl-terminal 26 amino acids which contain the CKII phosphorylation site, PAb 421 eptitope and negative regulatory region for DNA binding. This region of the protein is replaced by a short set of unique amino acids as indicated. Adapted from Han and Kulesz-Martin, 1992.

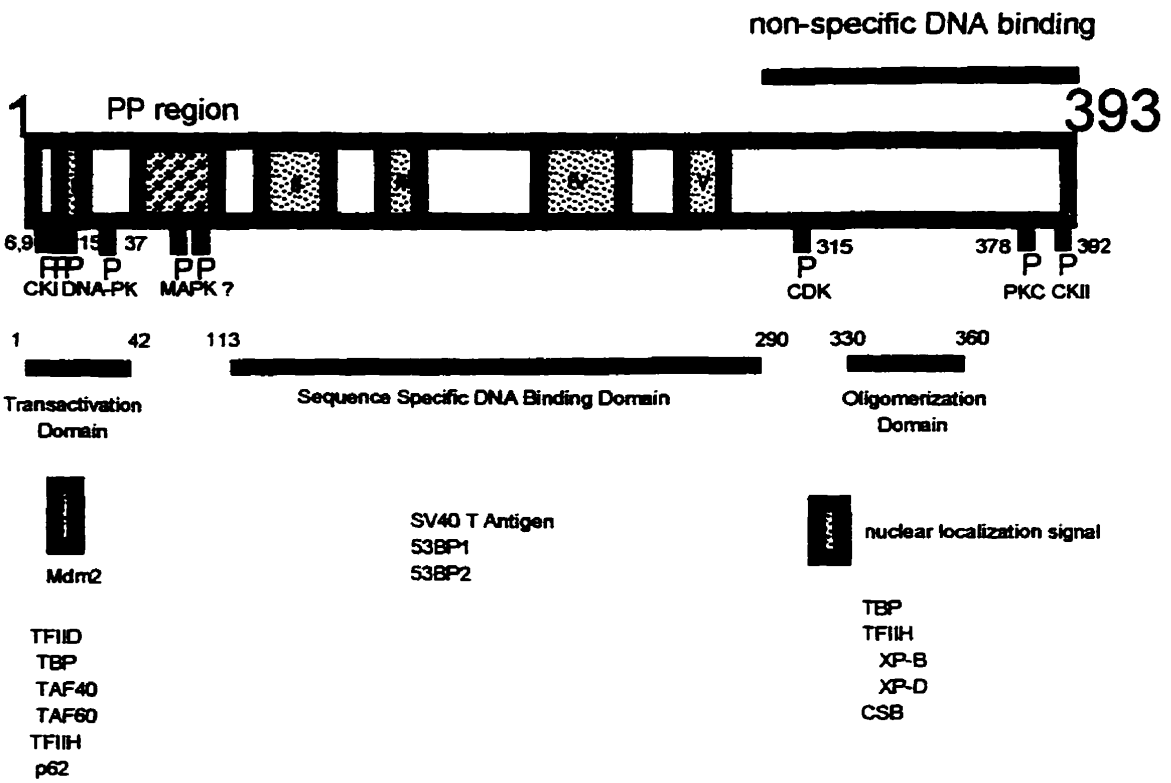

Figure 2: Functional Domains of p53 protein. Conserved domains are represented by numbered hatched boxes. Location of various domains, approximate binding location of p53 binding proteins, and phosphorylation sites as discussed in text are shown, where known. Adapted from Ko and Prives, 1996, Steeganga et al., 1996, and Mowat, 1998.

1993) and TFIIB (Horikoshi et al. 1995; Liu and Berk, 1995). As will be outlined in a subsequent section these binding events may be related to p53's transcriptional repression activities. Also contained within the amino terminus is a proline rich region (amino acids 61-94), which contains 5 repeats of PXXP. This region may play a role in signal transduction via SH3 domain binding activity, and be required for growth inhibition (Walker and Levine, 1996), through a signalling pathway involving the GAS1 gene product (Ruaro et al. 1997).

The central domain contains the sequence-specific DNA binding region (amino acids 100-300) (Vogelstein and Kinzler, 1992; Cho et al. 1994). The DNA binding surface is comprised of two β-sheets supporting two loops and a loop-sheet-helix motif (Cho et al. 1994). The two loops are connected by a zinc atom tetrahedrally coordinated between four residues, Cys176, His179, Cys238 and Cys242 (ibid). This region contains most of the mutations found in human tumours, which as outlined below may be related to loss of sequence-specific binding ability and transcriptional activation activity of the mutant proteins (Nigro et al. 1989; Hollstein et al. 1991). This region also contains four of the five evolutionarily conserved boxes identified by comparison between various vertebrate sequences ((Soussi et al. 1987; Soussi et al. 1990; Soussi and May, 1996), see Figure 2).

The carboxy terminus (amino acids 300-393) of p53 is a multifunctional region of the protein. It contains the oligomerization domain (Shaulian et al. 1995), for which the structure has been determined (Lee et al. 1994; Jeffrey et al. 1995; Clore et al. 1994). The carboxy terminus region may regulate the function of the sequence specific central DNA binding domain (Hupp et al. 1992; Wang and Prives, 1995). This function in turn may be

regulated by phosphorylation (Hupp et al. 1992; Wang and Prives, 1995). The C-terminal region also binds non-specifically to mismatched DNA or free DNA ends and can promote annealing of DNA single strands (Lee et al. 1995; Bakalkin et al. 1994; Bakalkin et al. 1995; Jayaraman and Prives, 1995). The C-terminal 75 amino acids also displays binding to damaged (Dnase I, ionizing radiation or restriction digested) DNA (Reed et al. 1995).

For a more detailed review on structural aspects of p53, the reader is directed to Arrowsmith and Morin (1996). Other exotic abilities have been assigned to p53 for which the regions have not been assigned. These include 3' to 5' exonuclease activity (Mummenbrauer et al. 1996), and the binding to and inhibition of action of the DNA repair protein Rad51 (Sturzbecher et al. 1996). In addition, N-terminal cleavage of p53 in response to DNA damage produces a 35 kD product which possesses protease activity (Molinari et al. 1996; Okorokov et al. 1997).

In addition to the transcriptional factors, p53 binds *in vitro* and *in vivo* to a number of proteins, including Mdm2 (Momand et al. 1992; Barak et al. 1993; Barak and Oren, 1992; Haines et al. 1994), Wilms Tumour (WT1) (Maheswaran et al. 1993), hsc70 (Hinds et al. 1987), spot-1 (Elkind et al. 1995), 53BP1 and 2 (Iwabuchi et al. 1994), Large T SV40 (Linzer and Levine, 1979; Lane and Crawford, 1979), and RPA (Dutta et al. 1993; Hinds et al. 1987) (See Table 1 and Figure 2). P53 can also bind to 5.8S RNA, which may allow it control over translation of various mRNAs including its own (Fontoura et al. 1997).

The p53 protein undergoes a number of post-translational modifications which have varied effects on its function. Phosphorylation occurs at multiple sites, clustered at the

Table 1: p53 binding proteins

Protein	Reference
TFIID	(Horikoshi et al. 1995; Liu and Berk, 1995)
TATA-binding protein (TBP)	(Seto et al. 1992; Liu et al. 1993; Martin et al. 1993; Truant et al. 1993; Farmer et al. 1996a)
TFIIB	(Horikoshi et al. 1995; Liu and Berk, 1995)
TBP associated factors	(Thut et al. 1995; Lu and Levine, 1995; Farmer et al. 1996a)
TAFII40/TAFII30	
TAFII60/TAFII70	
TFIIH	(Xiao et al. 1994; Wang et al. 1995; Leveillard et al. 1996)
RPA	(Dutta et al. 1993; Li and Botchan, 1993; Hinds et al. 1987)
human Rad51	(Stuerzbecher et al. 1996; Buchhop et al. 1997)
DP-1	(Sorensen et al. 1996)
CBP/p300	(Gu et al. 1997; Avantaggiati et al. 1997)
Wilm's Tumour (WT1)	(Maheswaran et al. 1993)
p53 BP1 and BP2	(Iwabuchi et al. 1994)
Ref-1	(Jayaraman et al. 1997)
Mdm2	(Momand et al. 1992; Oliner et al. 1993; Chen et al. 1993; Haines et al. 1994)
MdmX	(Shvarts et al. 1996)
Mdm2 related binding protein	(Hall and Milner, 1997)
hsc-70	(Hinds et al. 1987)
spot-1	(Elkind et al. 1995)
PACT (p53 assoc. cell protein - Testes)	(Simons et al. 1997)
E6-AP	(Maki et al. 1996; Huibregtse et al. 1993a,b)
S100b	(Baudier et al. 1992)
Casein kinase II	(Filhol et al. 1992)
c-Abl	(Goga et al. 1995)
trk tyrosine kinase	(Montano, 1997)
SV40 Large T antigen	(Linzer and Levine, 1979; Lane and Crawford, 1979)
Human Cytomegalovirus mtrII Oncoprotein	(Muralidhar et al. 1996)
IE2 86 kD protein	(Bonin and McDougall, 1997)
Adenovirus EIB 55 kD protein	(Sarnow et al. 1982; Zantema et al. 1985)
HPV E6	(Scheffner et al. 1990)
HBV Hbx	(Wang et al. 1994)
Epstein Barr Virus BZLF1	(Zhang et al. 1994)
Epstein Barr Virus EBNA-5	(Szekely et al. 1993)

amino terminus and spread over the carboxy terminus (Diagrammed in Figure2). To date, a number of kinases have been found to phosphorylate p53 either *in vitro*, *in vivo* or both (reviewed in (Steegenga et al. 1996)): CKI (Milne et al. 1992a; Knippschild et al. 1997), CKII (Herrmann et al. 1991; Meek et al. 1990), cdks (Cdc2 and cdk2) (Bischoff et al. 1990; Milner et al. 1990; Sturzbecher et al. 1990), DNA-damage activated kinase (Lees Miller et al. 1992), mitogen-activating protein kinase (MAPK) (Milne et al. 1994), stress-activated kinase (SAP/JNK) (Adler et al. 1997; Milne et al. 1995), PKC (Baudier et al. 1992; Hupp and Lane, 1994), PKA (Adler et al. 1997), raf1 (Jamal and Ziff, 1995), in rat a Large T activated kinase (Muller et al. 1993; Mueller and Scheidtmann, 1995), and recently, the kinase component (Cdk7-cycH-p36) of TFIIH (Lu et al. 1997). The conformation of p53 may be important for these phosphorylation events, as for example phosphorylation by PKA occurred more efficiently in native as opposed to GST-p53 constructs (Adler et al. 1997). Where appropriate, the effects of each phosphorylation event will be discussed in later sections.

Less is known about what enzymes dephosphorylate p53. Increased phosphorylation of p53 correlated with decreased PP2A activity due to small t antigen inhibition of PP2A in SV40 transformed cells (Scheidtmann et al. 1991b). Phosphatase 2A (PP2A) has been shown *in vitro* to dephosphorylate an *in vivo* phosphorylated p53 (Scheidtmann et al. 1991a). However more work needs to be done to determine whether PP2A or other phosphatases are required for p53 dephosphorylation.

p53 is subject to ubiquitination, which is probably involved in normal p53 degradation (Whitesell et al. 1997). Indeed, p53 protein accumulates in a ubiquitin defective

BALB/c 3T3 cell line (Chowdary et al. 1994). P53 is also subject to cleavage by the calcium-activated neutral protease, calpain, which may play a role in regulating p53 stability (Kubbutat and Vousden, 1997). p53 is acetylated both *in vitro* and *in vivo* in the C-terminal domain, which stimulates sequence-specific DNA binding activity (Gu and Roeder, 1997). p53 may also contain nuclear export signals as well as nuclear import signals, as import/export is carrier-mediated (ie. requires other factors besides signal sequences) (Middeler et al. 1997).

1.3 Biochemical and Functional Properties of p53.

p53 DNA binding:

p53 is thought to exert its tumour suppressor effect through transcriptional regulation, both as a transcriptional activator and a repressor. Transcriptional activation by p53 requires that it function as a sequence specific DNA binding protein. p53 is able to bind both double stranded and single stranded DNA (Steinmeyer and Deppert, 1988), and binds to ds DNA in a sequence specific manner (Kern et al. 1991b), to the p53 responsive element containing the consensus sequence 5'-RRRC(A/T)(T/A)GYYY-3' (R represents purine and Y pyrimidine; Funk et al. 1992; el Deiry et al. 1992). This p53 responsive site exists as two copies with a 0-13 bp spacer in between, allowing p53 to bind as a tetramer (Funk et al. 1992; el Deiry et al. 1992; Cho et al. 1994). DNA conformation is important for p53 sequence specific binding (Nagaich et al. 1997; Kim et al. 1997). The degree of bending of the recognition site is important, as more bent DNA will permit a stabler complex of p53 and its response element (Nagaich et al. 1997). The secondary conformation of the binding site

is also important, for example p53 protein in general binds best to a stem-loop structure with a single binding half site at the tip, different structures being favoured by different conformations of p53 (Kim et al. 1997).

Control over p53 sequence specific DNA binding occurs at several levels. Phosphorylation by several kinases (cdks, CKII) has been associated with enhanced binding in vitro (Wang and Prives, 1995; Hupp et al. 1992; Hao et al. 1996). Efficient DNA binding by p53 may also require acetylation. p53 is acetylated both in vitro and in vivo in the C-terminal domain, which stimulates sequence-specific DNA binding activity (Gu and Roeder, 1997).

Sequence specific binding by p53 is subject to redox modulation. Oxidation of p53 results in a protein which does not easily bind to consensus DNA *in vitro* and transactivate genes *in vivo*, but does not affect non-specific DNA binding (Parks et al. 1997). These authors suggest p53 may belong to a family of oxidative stress response factors which respond to hypoxia. Excess production of nitric oxide, while allowing rapid accumulation of p53 in the nucleus also results in a protein with changed conformation and significantly decreased specific DNA binding ability (Calmels et al. 1997). Interestingly, a recent paper suggests that p53 translocation to the nucleus after ionizing radiation may be controlled by free radical production (Martinez et al. 1997b). This translocation response could be blocked by the addition of a free radical scavenger (WR1065) (ibid). WR1065 did not affect p53 nuclear translocation after the addition other DNA damaging agents mitomycin C and doxorubicin, which do not produce free radicals (ibid).

An additional report suggests that p53 conformation and DNA binding activity may

also be affected by intracellular levels and redox activity of copper (Verhaegh et al. 1997). P53 has been shown to bind to the Cu^{1+} form of copper *in vitro*, and that interaction altered p53 conformation and DNA binding activity (Hainaut et al. 1995). In the later study, PDTC (pyrrolidine dithiocarbamate), a metal chelating compound which can act as either a antioxidant or prooxidant depending on the situation, altered p53 conformation from wild type and down-regulated p53 binding in several human cell lines (Verhaegh et al. 1997). This effect of PDTC on p53 correlated with it increasing the intracellular accumulation of copper, suggesting that copper ions may have a role in the physiological control of p53 function (ibid).

Transcriptional Activation by p53:

P53 appears to modulate the expression of a number of cellular genes important in cellular proliferation. The protein was originally identified as a transcriptional activator in 1990 (Raycroft et al. 1990; Fields and Jang, 1990). A large number of genes important for cellular growth contain p53 responsive elements within their promoter, and are activated by wild type p53. These include: GADD45 (Kastan et al. 1992), Mdm2 (Barak et al. 1993), $p21^{waf1/cip1}$ (el Deiry et al. 1993), Cyclin G (Okamoto and Beach, 1994; Zauberman et al. 1995), Bax (Miyashita et al. 1994b; Miyashita and Reed, 1995; Zhan et al. 1994a), IGFBP3 (Buckbinder et al. 1995; Bourdon et al. 1997), PCNA (Shivakumar et al. 1995; Hao et al. 1996), A28-RGS14 (regulator of G proteins signalling family member, (Buckbinder et al. 1997)), BTG2, a potential tumour suppressor (human) (Rouault et al. 1996), and hepatocyte growth factor/scatter promoter (Metcalfe et al. 1997). The list of potential p53 responsive

genes has expanded very quickly. Witness the identification of 55 candidate genes with p53 responsive elements, using the technique which identified IGF-BP3 (Buckbinder et al. 1995; Bourdon et al. 1997). A list of known and potential p53 responsive genes are given in Table 2.

Transcriptional activation of target genes by p53 is required for its growth suppressive functions (Crook et al. 1994; Pietenpol et al. 1994). Indeed, most human and mouse p53 mutants which are unable to function as wild type protein can no longer activate transcription (Raycroft et al. 1991; Raycroft et al. 1990). Many of these responsive genes have functions in cell growth regulation which are pertinent for the growth suppressive functions of p53. For example, activation of $p21^{waf1/cip1}$ and GADD45 may be important for p53 regulation over G1 arrest in response to DNA damage (to be discussed) (Morgan and Kastan, 1997; Zhan et al. 1994b). P53 activation of the Mdm2 gene as outlined below is important for the regulation of p53's own activity (Momand et al. 1992; Oliner et al. 1993; Chen et al. 1994). Bax gene activation may be important for induction of p53 mediated apoptosis (Miyashita et al. 1994b; Miyashita and Reed, 1995; Zhan et al. 1994a; McCurrach et al. 1997). Further, activation of various p53 targets may be important for treatment regimens which work through p53 pathways. For example, GML (glycosyl-phosphatidylinositol (GPI)-anchored molecule-like protein) is a p53 inducible gene with homology to a family of GPI anchored membrane proteins (Furuhata et al. 1996). On its own it suppresses growth in esophageal cancer cells in culture, and its expression correlated with sensitivity to various anti-cancer drugs (ibid).

Table 2: P53 Transcriptional Targets:

Transcription Activated	Reference:
Human epidermal growth factor receptor (EGF-R)	(Deb et al. 1994; Ludes-Meyers et al. 1996; Sheikh et al. 1997)
p21/Waf1/Cip1	(el Deiry et al. 1993)
Mdm2	(Barak et al. 1993; Juven et al. 1993; Otto and Deppert, 1993)
GADD45	(Kastan et al. 1992)
Bax	(Miyashita et al. 1994b; Miyashita and Reed, 1995; Zhan et al. 1994a)
Rb	(Osifchin et al. 1994)
PCNA	(Shivakumar et al. 1995; Hao et al. 1996)
TGF-alpha	(Shin et al. 1995)
IGFBP3	(Buckbinder et al. 1995; Bourdon et al. 1997)
Thrombospondin	(Dameron et al. 1994)
Cyclin G	(Okamoto and Beach, 1994; Zauberman et al. 1995; Bates et al. 1996)
BTG2	(Rouault et al. 1996)
A28-RGS14	(Buckbinder et al. 1997)
hepatocyte growth factor/scatter promoter	(Metcalfe et al. 1997)
GML (glycerol-phosphatidylinositol (GPI) anchored membrane like protein)	(Furuhata et al. 1996)
KILLER/DR5	(Wu et al. 1997)
PAG608	(Israeli et al. 1997)
Transcriptional Repression:	
c-myc	(Ragimov et al. 1993)
c-fos	(Ginsberg et al. 1991; Santhanam et al. 1991;
	Kley et al. 1992)
b-myb	(Lin et al. 1992)
IL-6	(Santhanam et al. 1991)
DNA Polymerase alpha	(Lin et al. 1992)
Topoisomerase II	(Sandri et al. 1996)
hsp70	(Agoff et al. 1993)
Bcl-2	(Miyashita et al. 1994a,b)
Rb	(Shiio et al. 1992; Osifchin et al. 1994)
Actin	(Ginsberg et al.1991; Santhanam et al.1991)
Muscle Creatine Kinase	(Weintraub et al. 1991)
MHC	(Santhanam et al. 1991)
SV40 early region	(Jackson et al. 1993)
HSV type 1 UL9 promoter	(Deb et al. 1992; Jackson et al. 1993)
Human Cytomegalovirus major immediate early promoter enhancer	
LTR Rous sarcoma and human T cell Lymphoma virus	

Control over Transcriptional Activation and p53 Function:

The relationship between phosphorylation, DNA binding and transcriptional activity is far from simple, and it has been suggested that phosphorylation may have different effects depending on the cellular environment and response element involved (Lohrum and Scheidtmann, 1996). Phosphorylation at the C-terminus by CKII has been shown to activate p53 binding activity *in vitro* (Hupp et al. 1992). However, some mutants at the CKII site can still bind the consensus site *in vitro* (Rolley and Milner, 1994). Mutation of serine to alanine at the CKII site abolishes the growth suppressor activity of p53 when expressed in non-transformed cell lines (Milne et al. 1992b). Most *in vivo* studies have failed to find altered DNA binding or transactivation activities after mutation of the CKII phosphorylation site (Crook et al. 1994; Fiscella et al. 1994; Fuchs et al. 1995). A recent report suggests that these discrepancies might be explained by the environment in which p53 is expressed. Mutants at a number of phosphorylation sites including the CKII site on p53 acted as wild type in standard transient transfection experiments in NIH 3T3 cells (Hao et al. 1996). When the cells were arrested at G1 by contact inhibition (where wild type p53 is inactive), only one mutant (Ser389 to Glu) was functional (ibid). In contrast, substitution to Ala resulted in loss of binding and transcriptional activity (ibid). Specific phosphorylation of Ser 389 by overexpression of Cyclin E activated wild type function in all but the Glu mutant (Hao et al. 1996). One also sees altered DNA binding after phosphorylation by the S and G2 phase cdks (Wang and Prives, 1995), however mutant p53 with a Ser 312 to Ala substitution in both humans and murine systems did not alter p53 transcriptional activities or the ability to suppress focus formation (Slingerland et al. 1993;

Fuchs et al. 1995; Marston et al. 1994). Phosphorylation by DNA-PK has previously been reported to be dispensable for p53 induced growth arrest or transactivation using DNA-PK null cells (Rathmell et al. 1997). However, a recent report suggest that phosphorylation by DNA-PK may be important for p53 response to DNA damage by relieving Mdm2 regulation of p53 (Shieh et al. 1997). Together this suggests that in some specific circumstances (DNA damage response or quiescence), phosphorylation may be more important for control over p53 transcriptional function.

Further control over p53 transactivation occurs during interaction with different binding proteins, the two best characterized being Mdm2 and CBP/p300. Mdm2 (murine double minute) is a gene amplified and overexpressed in human sarcomas (Oliner et al. 1992; Ladanyi et al. 1993). Mdm forms a tight complex with p53, concealing the transactivation domain from its targets, which blocks p53 transcription activation activities (Momand et al. 1992; Oliner et al. 1993; Chen et al. 1993; Haines et al. 1994). The Mdm gene is also a target for p53 induced transactivation (Barak et al. 1993; Juven et al. 1993; Otto and Deppert, 1993), resulting in a negative feedback loop (Wu et al. 1993). Overexpression of Mdm2 can inhibit p53 induced G1 arrest and apoptosis (Chen et al. 1994; Chen et al. 1996). Further, overexpression of Mdm2 allows for tolerance of high p53 levels in cell, in part explaining why wild-type expression still occurs in some tumour tissues (Blaydes et al. 1997). In addition, Mdm2 directs p53 to proteosome degradation (Haupt et al. 1997; Kubbutat et al. 1997). Control over Mdm2 binding may be regulated through the DNA-PK phosphorylation site. Phosphorylation by DNA-PK after DNA damage impairs the ability of Mdm2 to bind and inhibit p53-dependent transactivation (Shieh et al. 1997). Phosphorylation by other

kinases such as Cdc2 or CKII does not affect Mdm2 binding (Bond et al. 1994). P53 also interacts with other Mdm family members such as MdmX, which specifically interacts with the N-terminus of p53 and can inhibit the transactivation domain of p53 during cotransfection (Shvarts et al. 1996). However, expression of mdmX mRNA is not affected by UV suggesting it may regulated in a different manner than Mdm2 (ibid).

p53 transcriptional activation activity can be mediated by interaction with the transcription factor CBP/p300 (Avantaggiati et al. 1997; Gu et al. 1997; Lill et al. 1997). CBP/p300 and p53 interact both *in vitro* and *in vivo* (Gu et al. 1997; Avantaggiati et al. 1997), in a complex which colocalizes to the nucleus (Lill et al. 1997). Binding of p53 and CBP enhances p53's transcriptional activation activity (Avantaggiati et al. 1997; Gu et al. 1997; Scolnick et al. 1997). For example, CBP enhanced p53 activation of the Mdm2 gene in Saos-2 cells (Gu et al. 1997). However, CBP/p53 binding reduces activation of $p21^{waf1/cip1}$ and bax promoters (Lill et al. 1997). Also, a dominant negative form of p300 prevented transactivation by p53, and prevented p53 induced G1 arrest and apoptosis (Avantaggiati et al. 1997). E1A (which binds p300) can disrupt p53-induced activation of $p21^{waf1/cip1}$ and Bax, plus suppress cell cycle arrest and apoptosis (Lill et al. 1997). The p300/CBP binding region of E1A is capable of inhibiting p53 transactivation functions (Somasundaram and El-Deiry, 1997). Addition of exogenous CBP or p300 could alleviate E1A inhibition of p53 mediated transcription (ibid). Further, an E1A mutant unable to bind Rb but able to bind to CBP/p300 could still repress p53 dependent cell cycle arrest (ibid). CBP and p53 complex to form a repressor for human hsp70 promoter (Agoff et al. 1993). Together this suggests that CBP/p300 and p53 interaction is important for regulation of transcription by p53, and

checkpoint control.

Other proteins that may affect p53 DNA binding/transcriptional activities are the p53 binding proteins BP1/BP2, the Wilms Tumour Gene product, and Ref-1. BP1 has no known homology to other proteins, while BP2 contains two ankyrin repeats and a Src homology 3 domain (Iwabuchi et al. 1994). Both proteins bind to p53 in the sequence specific DNA binding portion of the central domain, preventing p53 binding to its consensus sequence (Iwabuchi et al. 1994). Both also bind exclusively to wild type p53 (Iwabuchi et al. 1994). The p53/BP1 complex also interacts with a third protein, p202. P202 is a BP1 binding protein (Datta et al. 1996). P202 will inhibit transcriptional activation by p53, which can be overcome by binding of p202 by a segment of BP1 (98 aa) (Datta et al. 1996).

Interaction between the Wilms tumour suppressor protein WT1 and p53 modifies their ability to transactivate target genes (Maheswaran et al. 1993). WT1 on its own acts as an transcriptional activator of the early growth response gene (EGR1). Bound to p53, WT1 converts to a transcriptional repressor while also enhancing transcriptional activation abilities of p53 (ibid). The Ref-1 protein, which regulates the redox state of proteins and functions as a DNA repair (A/P) endonuclease also binds and stimulates p53 transactivation (Jayaraman et al. 1997).

Transcriptional Repression by p53:

A second potential mechanism for p53 control over cell proliferation may be its transcriptional repressor abilities. p53 represses a wide variety of cellular and viral promoters. These include: c-fos (Ginsberg et al. 1991; Santhanam et al. 1991; Kley et al.

1992); c-myc (Ragimov et al. 1993); b-myb (Lin et al. 1992); IL-6 (Santhanam et al. 1991); DNA Polymerase alpha (Lin et al. 1992); topoisomerase II alpha (Sandri et al. 1996); hsp70 (Agoff et al. 1993); and a number of viral promoters. These targets are summerized in Table 2.

Unlike transcriptional activation, repression by p53 does not require direct binding to DNA (Deb et al. 1992; Mack et al. 1993). Instead, p53 probably acts through interaction with the basal transcription factors (Ragimov et al. 1993). The amino terminus of p53 has been shown to directly interact with members of the Transcription factor (TF) TFIID complex (Horikoshi et al. 1995; Liu and Berk, 1995), such as the TATA box binding protein (TBP) (Seto et al. 1992; Liu et al. 1993; Martin et al. 1993; Truant et al. 1993; Farmer et al. 1996a), TFIIB (Horikoshi et al. 1995; Liu and Berk, 1995), and TBP-associated factors TAFII40/TAFII31 and TAFII60/TAFII70 (Drosophila/human) (Thut et al. 1995; Lu and Levine, 1995; Farmer et al. 1996a), and the TFIIH complex (Xiao et al. 1994; Wang et al. 1995; Leveillard et al. 1996).

Repression also requires the presence of a TATA box within the target gene's promoter (Mack et al. 1993). Past work suggests that repression of basal transcription by p53 involved interaction with TBP (Seto et al. 1992; Ragimov et al. 1993). However, p53 failed to repress expression of a TATA-promoter stimulated by overexpression of TBP *in vivo* (Farmer et al. 1996b). Overexpression of TBP also failed to rescue from p53 induced repression of activated transcription (ibid). Mutation of TAF binding site on p53 did ablate p53 repression, suggesting p53-TBP interaction is not sufficient for repression, but requires interaction with other factors such as the TAFs (ibid). Indeed, excess of TFIIB or TFIID can

prevent p53 induced repression of target promoters, suggesting p53 may work either directly or indirectly through these factors (Liu and Berk, 1995). P53 mediated transcriptional repression can also be overcome *in vitro* by TFIIH (Leveillard et al. 1996). Phosphorylation of p53 may or may not affect the protein's transcriptional repression functions. Preventing phosphorylation at the CKII site (serine 386/389) by altering the serine to alanine does not affect transcriptional activation, but it does reduce the mutant's ability to repress the c-fos promoter (Hall et al. 1996). There is preliminary evidence that p53 can also repress Pol III directed transcription *in vitro* and *in vivo* (Chesnokov et al. 1996). These authors found that the amino terminus of p53 binds to TFIIIB, suggesting a similar mechanism to that seen with Pol II (ibid).

Transcriptional repression by p53 may be an important component for its apoptotic functions. Expression of either Bcl-2 or the adenovirus E1B 19-kD proteins blocks p53 mediated apoptosis, and suppresses transcriptional repression by p53 (Shen and Shenk, 1994; Sabbatini et al. 1995). P53 negative regulation of the topoisomerase II alpha promoter could potentially function as part of p53 apoptosis induction pathway (Sandri et al. 1996; Wang et al. 1997). Topoisomerase II alpha catalyses changes required for disentanglement of newly-replicated sister chromatids (ie. relief of torsional stress) (ibid). If there is no topoisomerase II activity, it results in the creation of chromosomal breakage during anaphase (ibid). It is interesting to note that the primary target for antineoplastic drugs (etoposide, doxorubicin etc) is thought to be topoisomerase II (Sandri et al. 1996).

1.4 p53 Mutations in Cancer

Mutation of the p53 gene occurs in approximately 50% of all human cancers (Hollstein et al. 1991; Florenes et al. 1994; Greenblatt et al. 1994; Hollstein et al. 1996a; Hollstein et al. 1996b; Beroud et al. 1996; Hainaut et al. 1997). Interestingly, the majority of these mutations are found in the central DNA binding domain. Forty percent of mutations occur in "hotspots" within conserved region III (Arg 175), IV (Gly245, Arg248, Arg249), and V (Arg 273 and Arg 282) (Nigro et al. 1989; Hollstein et al. 1991). A large number of these mutations occur at CpG dinucleotides (includes 175, 248 and 273), suggesting these hotspots may be influenced by cytosine methylation (Denissenko et al. 1997). CpG may be a preferential target for exogenous chemical carcinogens, as *anti*-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE) preferentially forms adducts with methylated CpG (ibid.).

Since mutation of p53 occurs primarily in the DNA binding region, it might suggest that abrogation of p53 sequence-specific binding and transcriptional activation is important for inactivation of wild type p53 function. Indeed, many human p53 mutants have lost their sequence-specific DNA binding ability (Kern et al. 1991b; Bargonetti et al. 1992; Kern et al. 1991a). These mutants usually fail to trigger transcription of target genes (Kern et al. 1991b), resulting in loss of tumour suppressor activity. However, this is not the entire story since some p53 mutants while unable to induce growth arrest still retain the ability to bind sequence specifically to DNA (Pietenpol et al. 1994; Zhang et al. 1993).

This leads to the conclusion that all mutations of p53 are not equal. Deletion of one allele with missense mutation of the second is the most common mutation seen in human

cancer, suggesting at least some mutant p53 proteins function by abrogating wild-type p53 function, or gain new oncogenic functions (Iwamoto et al. 1996). Thus, mutation of p53 may in some cases not only disable wild type function, but also confer a gain of function upon the mutant allele. One example of this is the effect missense mutants (Trp248, Ser249, His273) have on T cell surface receptor expression (Iwamoto et al. 1996). In this case there was enhanced frequency of TCR mutants after graded doses of X-radiation in lymphocytes containing p53 missense mutants compared to null p53 parent and wild-type p53 lymphocytes (Iwamoto et al. 1996). Mutant p53 expressed in cell lines which lack p53 resulted in enhanced tumourigenic potential in nude mice ((10)3 cells) or enhanced plating efficiency in agar cell culture (SAOS-2 cell line) (Dittmer et al. 1993). Mtp53 alleles could also enhance expression of the CAT gene under the multi-drug resistance enhancer promoter element (ibid).

1.5 P53 in DNA Repair and Damage Response

There is some suggestion that p53 may "sense" unusual structures of DNA, which may be important for DNA damage response. P53 protein exhibits a rapid accumulation in the nucleus (Sjoeblom and Laehdetie, 1996), and sequence specific binding in response to radiation or chemically induced DNA strand breaks (Lu and Lane, 1993; Tishler et al. 1993; Nelson and Kastan, 1994). Induction of p53 after DNA damage may in addition be controlled through protein kinases such as DNA-PK. P53 is phosphorylated at the DNA-PK site (serine 15) after γ irradiation induced DNA damage in LNCaP cells, which impairs the

ability of Mdm2 to bind and inhibit p53 function (Shieh et al. 1997). In vitro phosphorylation of p53 by DNA-PK also attenuated the interaction with Mdm2, relieving p53 from Mdm2 downregulation (ibid).

P53 can form highly stable (half life of >2 hours) complexes as a tetramer with DNA insertion/deletion mismatches (Lee et al. 1995). A portion of the C-terminal domain (amino acids 361-382) can recognise and bind to staggered single stranded ends of DNA, which stimulates sequence-specific and non-specific binding of the core domain (Bakalkin et al. 1995; Jayaraman and Prives, 1995; Selivanova et al. 1996). Binding of p53 protein to single stranded DNA ends catalyses DNA renaturation and DNA strand transfer (Bakalkin et al. 1994; Reed et al. 1995). For example, in thyroid cells wild type p53 enhances DNA joining activity (rejoining of plasmid with luciferase reporter) (Yang et al. 1997). P53 induced complementary strand annealing can be blocked by the binding of CKII to p53, suggesting a possible regulatory mechanism (Filhol et al. 1996). In addition, p53 binds to four way Holliday Junctions, and addition of p53 increases resolution of Hol_{75} DNA by T4 endonuclease VII and T7 endonuclease I (two enzymes which cleave these junctions) (Lee et al. 1997). All of these functions may be important for p53's proposed role in the maintenance of genomic stability.

1.6 P53 and Genomic Stability

Cancer is a disease of genetic instability. Maintaining genomic stability has been proposed as one major function of p53 in preventing carcinogenesis. No doubt p53 plays a role in this, but this role may be more or less important depending on the particular cell types

and conditions. Germline inactivation of p53 on it's own does result in tumour formation in both humans (Li-Fraumeni patients) and mice (Donehower et al. 1992; Jacks et al. 1994; Malkin, 1994). P53 loss is also associated with increased gene amplification (Livingstone et al. 1992; Yin et al. 1992), including the Met and Myc oncogenes (Rong et al. 1995; Tainsky et al. 1995; Fukasawa et al. 1997). Some p53 mutants may even have an activity which promotes mutations. One group looking at the mutation rate of a SV40-based shuttle vector system in Li-Fraumeni fibroblasts. This group noted that deletions of the Large T antigen gene occurred in cells possessing a mutant p53 allele, but not in p53 null Saos-2 cells (Liu et al. 1996). This effect of mutant p53 could result in a high rate of chromosomal instability and allelic loss wild type p53 observed during immortalization of the fibroblasts (ibid). Together, these results indicate that increased aneuploidy in cells lacking functional p53 could promote other tumour suppressor/oncogene mutations by chromosome loss or increased onocogene expression due to a gain of chromosome number, thus promoting the transformation process.

It should be stressed that mutation of p53 is not the whole story, and in fact p53 independent/mutagenic carcinogenic pathways must exist. An inverse correlation between replication error (RER)-prone cells (human colorectal) and p53 status was found in one study (Cottu et al. 1996). All RER+ were wild type for p53, however 15/17 RER- were mutant. In this case mismatch repair deficiency provides a p53 independent pathway for development of colorectal cancers. (Cottu et al. 1996). Another study on human sporatic colorectal cancers found tumours were more likely to have p53 mutation if they possessed no or minimal genetic instability (Kahlenberg et al. 1996). A test of the mutation frequency

of the hprt locus in p53null pre B-cells mice (where B-cells normally are radiation ultrasensitive), found a high number of hprt mutants in null cells, but a normal mutation rate (Griffiths et al. 1997). This suggested there was increased survival because p53 was missing and not a p53 dependent increase in the mutation rate (Griffiths et al. 1997).

1.7 p53 and Development

p53's role in development is yet undefined. There is a fair body of evidence to suggest regulation of p53 expression is essential for normal development, and inactivation of p53 may be required to allow for differentiation. Peak expression of p53 occurs during murine development in rapidly proliferating less differentiated cells, in a manner suggestive of a role in differentiation (Gottlieb et al. 1997; Komarova et al. 1997). p53 activity *in vivo* is also determinant of radiation and drug sensitivity in early embryos (Komarova et al. 1997). During prophase of meiosis in rat spermatogenesis, maximal levels of p53 are seen in the end of zygotene and beginning of pachytene (stages XIII-I), which is the time of recombination (Sjoblom and Lahdetie, 1996). The splice variant Asp53 is preferentially expressed in more mature keratinocyte cells. This suggests a role for this splice form in keratinocyte differentiation (Rehberger et al. 1997b; Rehberger et al. 1997a). Further, expression of p53 in undifferentiated embryonic stem cells decreases and shifts conformation to the non-functional form as differentiation occurs (Sabapathy et al. 1997), suggesting that inactivation of p53 function is important for differentiation and development. In support of that theory, $Mdm2^{-/-}$ knockout mice are not viable, with embryonic lethality around implantation (Luna et al. 1995; Jones et al. 1995). Backcross into a p53 null background

rescues these mice from lethality, suggesting Mdm2 control over inappropriate p53 function is essential during development (ibid). In contrast to the above, p53 $^{-/-}$ mice develop normally (Donehower et al. 1992), suggesting expression is not necessary for overall normal development in mammals. Interestingly, inhibition of p53 activity by microinjection of a dominant negative human p53 into *Xenopus* embryos, or overexpressing the *Xenopus* version of the Mdm2 p53 negative regulator (Xdm-2), prevented differentiation of Xenopus early blastomeres (Wallingford et al. 1997). The embryos instead formed tumour like cellular masses, suggesting that in *Xenopus* wild type p53 expression is essential for normal development (ibid). Thus, the role p53 plays in development may be more complex than simply repression, and remains to be determined.

1.8 p53 Family Members

Until recently, p53 existed as an "orphan" without a related gene family. This has changed with the recent discovery of two proteins with homology to p53. The first is the human gene encoding p73, a protein which shares sequence homology to p53 (Kaghad et al. 1997). The p73 gene maps to a region in chromosome 1p36, which is frequently deleted in a number of cancers including neuroblastoma (Kaghad et al. 1997). P73, like p53, produces two splicing variants differing at the C-terminus (Kaghad et al. 1997). The p73 also appears to share growth inhibiting and apoptotic abilities similar to p53. For example, p73 stimulates $p21^{waf1\backslash cip1}$ production and suppresses colony formation when transfected into SK-N-AS cells (Kaghad et al. 1997). P73 can also stimulate apoptosis in a manner similar to p53 (Jost et al. 1997). However, unlike p53, p73 is not produced in response to UV induced DNA

damage suggesting it may behave somewhat differently than p53 (ibid).

The second gene, termed KET was isolated from rat cDNA library and exhibits approximately 38% homology with human and rat p53, the strongest homology (75%) occurring in the evolutionarily conserved regions (Schmale and Bamberger, 1997). Not much is known on KET function. KET is expressed during embryonic development, suggesting that it may complement or potentially compensate for the functions of p53 in p53 -/- mice during development (ibid).

2 Growth Inhibitory Functions of p53

2.1 Apoptosis

p53 is an important mediator of apoptosis in many cell types (Yonish-Rouach et al. 1991; Shaw et al. 1992; Clarke et al. 1993). This apoptosis occurs in response to various stimuli, including overexpression of viral or cellular oncogenes such as adenovirus E1A (Debbas and White, 1993; Lowe and Ruley, 1993), myc (Wagner et al. 1994), E2F (Wu and Levine, 1994), DNA damage (Clarke et al. 1993; Lotem and Sachs, 1993), and the removal of growth factors (Johnson et al. 1993; Gottlieb et al. 1994). Wild type p53 is required for apoptosis induced by many chemotherapeutic drugs and ionizing radiation, indicating p53 status may be important for potential outcome in treatments (Clarke et al. 1993; Lee and Bernstein, 1993; Lotem and Sachs, 1993). However, it is important to note that apoptosis can occur without the action of p53 (Yamamoto et al. 1996; Clarke et al. 1993; Lowe et al. 1993). For example, induction of thymocyte apoptosis is both p53 dependent and

independent. P53 null thymocytes were resistant to induction of apoptosis by radiation or etoposide (agents which cause DNA strand breaks). They still had normal sensitivity to glucocorticoid and calcium induced apoptosis, demonstrating that other apoptotic pathways which are not dependent on p53 exist (Clarke et al. 1993).

P53 mediated apoptosis can be overcome by the expression of various survival factors, including Bcl-2, Adenovirus E1B 55kd, and Interleukins 3 and 6 (Debbas and White, 1993; Yonish-Rouach et al. 1993; Clarke et al. 1993; Gottlieb et al. 1994; Canman et al. 1995). For example, induction of apoptosis in M1 myeloid leukemic cells by wild type p53 expression is inhibited by IL-6 expression (Yonish-Rouach et al. 1991; Yonish-Rouach et al. 1993). p53 may influence cytokine-elicited cellular signalling through Janus kinase-signal transducers and activators of transcription (Jak-STAT) pathway. Overexpression of wtp53 (val135 mutant) in cells resulted in reduced response to IL-6, and loss of STAT3 and STAT5, suggesting IL-6 triggered masking of these factors (Rayanade et al. 1997).

Several p53 transcriptional targets are associated with p53 apoptotic activities, the best characterized being Bax and IGF-BP3 (insulin-like growth factor binding protein 3). p53 transcriptionally activates Bax (Bcl-2 homolog (Miyashita and Reed, 1995)), an apoptotic promoter which binds to Bcl-2 and blocks its ability to prevent apoptosis (Miyashita et al. 1994b; Miyashita and Reed, 1995; Zhan et al. 1994a; Merchant et al. 1996). Loss of Bax expression is important for survival in some cells, such as in E1A expressing primary fibroblasts where apoptosis is dependent on p53 (McCurrach et al. 1997). Bax may also act as an effector of p53 mediated apoptosis in response to chemotherapy and may contribute to a p53 pathway which suppresses the oncogenic process (McCurrach et al.

1997). This response also involves p53 cofactors which act with Bax for apoptotic response (ibid). However, Bax/p53 is not always sufficient for radiation induced apoptosis in some cell types. The SW626 human ovarian cancer cell line does not undergo apoptosis in response to either upregulated p53 (val135) or Bax (Strobel et al. 1997). IGF-BP3 is also transcriptionally activated by p53 and can block the insulin growth factor signalling pathway by binding and preventing IGF from interacting with its receptor (Buckbinder et al. 1995; Bourdon et al. 1997). Other proteins that may be involved are the Gas2 and Gas3 genes associated with p53 dependent apoptosis (Brancolini et al. 1997), A28-RGS14 (regulator of G proteins signalling family member, (Buckbinder et al. 1997)), PAG608 (nuclear zinc finger protein, (Israeli et al. 1997)), or caspases (Interleukin 1β Converting Enzyme-like proteases), which is proteolyticaly activated during p53 mediated apoptosis in BRK cells (transfected with E1A and val135) (Sabbatini et al. 1997). Apoptosis in these cells required transcriptionally active p53 and was inhibited by a caspase inhibitor (Sabbatini et al. 1997).

In summary, a number of factors (presence/absence of growth factors, DNA damage, or activated oncogenes) are involved in the choice whether p53 directs a cell to enter cell cycle arrest or apoptosis. Apoptosis and growth arrest clearly are separable activities of p53. For example, M1 myeloid leukemic cells do not arrest in response to overexpression of wild type p53, but still undergo apoptosis (Yonish-Rouach et al. 1993). J3D cells expressing HPV16 E7 and tsp53 do not arrest at G1 when wild type conformation is induced, but still exhibit same level of p53 induced apoptotic death (Wang et al. 1996). Thus, the presence of an activated oncogene pushing cells to divide in an unsuitable environment (eg. Lacking growth/survival factors), or the presence of overwhelming DNA damage may promote p53

apoptotic activities over growth arrest (Levine, 1997).

2.2 p53 Control over Cell Cycle Progression

p53 protein is stabilized in response to DNA damage and other stresses, inducing growth arrest (Kastan et al. 1992; Lu and Lane, 1993). The ability of p53 to inhibit cell growth at G1/S is due in part to it's ability to bind specific DNA sequences and activate transcription of target genes involved in cell cycle regulation such as the gene encoding the cell cycle inhibitor $p21^{waf1\backslash cip1}$. P53's function in G1/S control has been well characterized and will be dealt with in a later section. Much less is known about the possible function(s) of p53 at G2/M. Before addressing this, a brief discussion of cell cycle control at G2, and checkpoints over the cell cycle is in order.

General Cell Cycle Control at G2/M:

In Eukaryotes, progression through the cell cycle is controlled by a family of cyclin dependent kinase (cdks) which form an active complex with cyclins (For a review of cell cycle, please see (Forsburg and Nurse, 1991; Norbury and Nurse, 1992; King et al. 1994; Lew and Kornbluth, 1996; Fisher, 1997). Cyclins were originally identified in sea urchin eggs as a group of periodic proteins which are renewed beginning at the start of the cell cycle, and destroyed at mitosis (Evans et al. 1983). Each cdk must bind to its appropriate cyclin partner before the kinase can become active (Brizuela et al. 1989).

Control over the transition from G2 into mitosis is mediated by Cdc2 kinase and the cyclin members A, B1 and B2 (Sherr, 1993; Pines and Hunter, 1992). Cdc2 kinase was the

first cyclin dependent kinase identified from work done on both *Saccharomyces cerevisae* (Cdc28) and *Schizosaccharomyces pombe* (Forsburg and Nurse, 1991; Lew and Kornbluth, 1996). At present Cdc2 kinase has been the only cell cycle kinase found to be indispensable for passage through G2 and into mitosis (for review see (King et al. 1994; Grana and Reddy, 1995)). Cdc2 and cyclin B1 also comprise the Maturation Promoting factor (MPF) required for progression from G2 and through meiosis in Xenopus oocytes (Dunphy et al. 1988; Coleman and Dunphy, 1994).

Cdc2 activation involves a precise series of phosphorylation and dephosphorylation steps (at residues Thr 14, Tyr 15, and Thr 161), which occur after binding to its cyclin partner B1 in S phase (Brizuela et al. 1989; Gautier et al. 1988; Smythe and Newport, 1992), for a review see (King et al. 1994) (Please see Figure 3). Immediately upon binding of Cdc2 and cyclin B1, Cdc2 is phosphorylated on Thr 161 by the cyclin dependent kinase activating (CAK) enzyme (Fesquet et al. 1993; Solomon et al. 1993; Poon et al. 1993). This phosphorylation event is required for Cdc2 activation (ibid; Gould et al. 1991). To prevent premature activation of Cdc2 at this point (S phase), the kinase complex is initially repressed by simultaneous phosphorylation of the Thr 14 and Tyr 15 residues by the Wee1 and Mik1 kinases, which phosphorylate Tyr 15 after cyclin binding (Heald et al. 1993; Parker et al. 1993). A third kinase, Myt1, can also down regulate Cdc2 by phosphorylating Tyr15, but preferentially acts on Thr 14 residue (Liu et al. 1997; Booher et al. 1997).

Activation of Cdc2 late in G2 to allow progression into mitosis requires the dephosphorylation of Thr 14 and Tyr 15. This event is thought to require the activation of Cdc25 phosphatases and the inhibition of Wee1 kinase activity (Gautier et al. 1991;

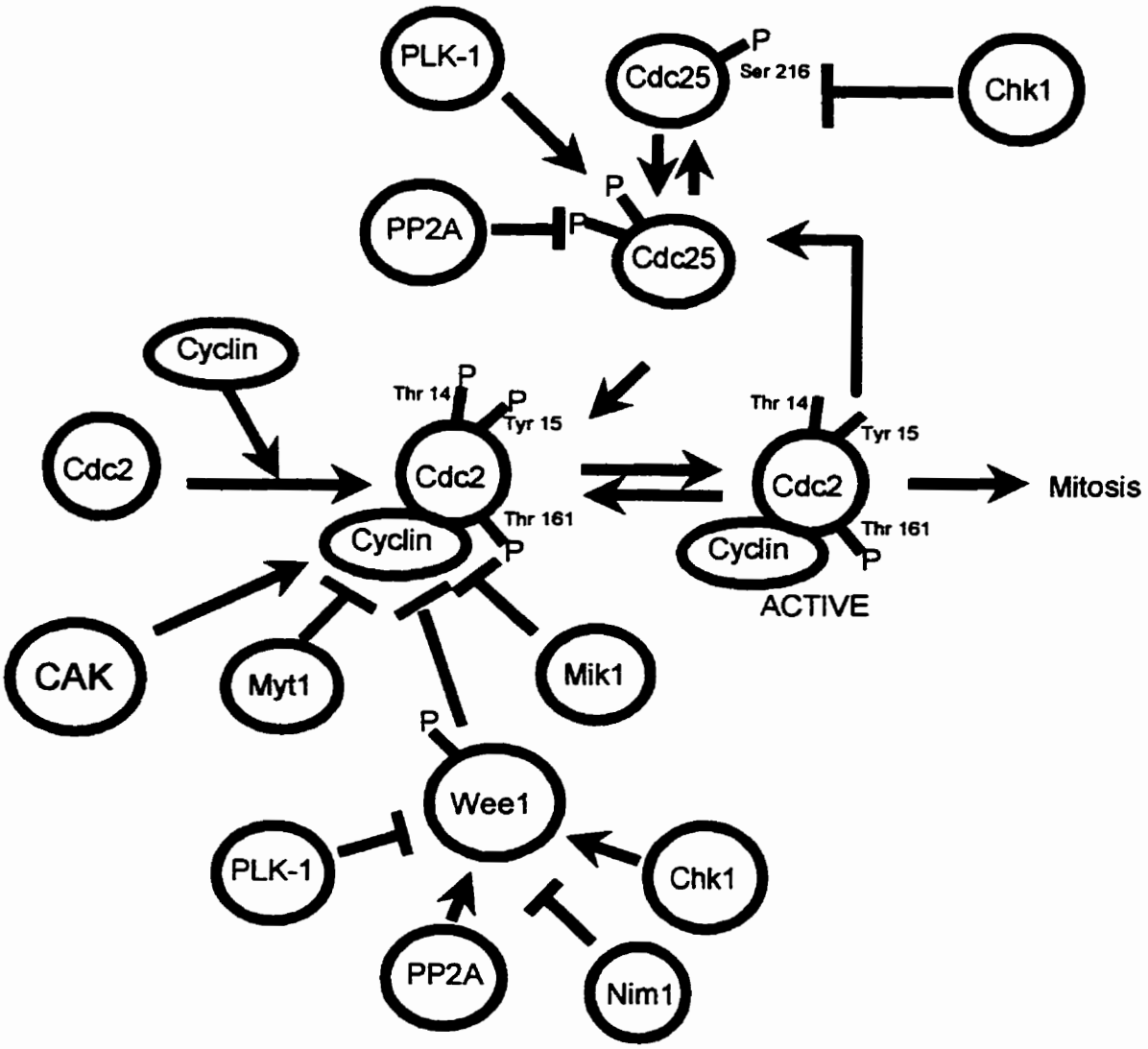

Figure 3: Control over Cdc2 kinase activity. The various control mechanisms as described in text are shown above.

Sebastian et al. 1993). The Cdc25 phosphatase family is comprised of three members, Cdc25 A, B and C (Galaktionov and Beach, 1991; Kakizuka et al. 1992; Nagata et al. 1991). Cdc25A is important for progression during G1 and does not appear to act later in the cell cycle (Hoffman et al. 1994; Jinno et al. 1994). Cdc25B, which is expressed through prophase, has the ability to dephosphorylate both tyrosine 15 and threonine 14 on Cdc2 and activate cytoplasmic Cdc2/Cyclin B, resulting in microtubule nucleation before mitosis (Gabrielli et al. 1996; Honda et al. 1993). Cdc25C is associated with G2 progression (Gautier et al. 1991; Hoffman et al. 1993; Sebastian et al. 1993).

Cdc25C shows an increase in phosphorylation at G2/M associated with its activation, and appears to exist in a positive feedback loop with Cdc2 kinase (Kumagai and Dunphy, 1992; Izumi et al. 1992; Hoffman et al. 1993; Izumi and Maller, 1995; Strausfeld et al. 1994). Cdc25C may also be activated by other cell kinases (Izumi and Maller, 1995). One such activating kinase is Plx1, originally isolated from Xenopus oocyte extracts (Kumagai and Dunphy, 1996). Plx1 is a member of the Polo kinase gene family which is required for normal mitosis (Llamazares et al. 1991). The human homolog for Plx has been implicated in centrosome maturation, proper mitotic progression and as part of a centrosome checkpoint control (Lane and Nigg, 1996).

In yeast Wee1 kinase is negatively regulated by the Nim1 kinase (Parker et al. 1993; Wu and Russell, 1993; Coleman et al. 1993). Nim1 in *S.pombe* may in turn be inhibited by the Nif1 protein, the absence of which causes smaller cell size after cell division (Wu and Russell, 1997). Working to control both Wee1 and Cdc25 phosphorylation events are phosphatases such as type-2A (PP2A). PP2A maintains Cdc25C in the inactive state and

Wee1 in the active unphosphorylated state, the consequence being maintenance of Cdc2 in a phosphorylated inactive state (Clarke et al. 1993; Kinoshita et al. 1993).

General Checkpoint Control:

Cells use a series of checkpoints to ensure completion of events during the cell cycle (such as DNA synthesis) and to delay progression into mitosis if circumstances require (for review see: Elledge, 1996; Nasmyth, 1996; Sherr, 1996; Collins et al. 1997). For example, growth checkpoints 1) ensure that DNA synthesis is completed before mitosis initiation (Weinert et al. 1994; Navas et al. 1995); 2) ensure that anaphase is not initiated until metaphase has been completed (Murray, 1992; Murray, 1995); and 3) prevent inappropriate DNA synthesis before the completion of mitosis (Cross et al. 1995).

The best characterized DNA damage response arrest points are found at G1/S, G2 and spindle assembly (Elledge, 1996; Nasmyth, 1996; Sherr, 1996; Collins et al. 1997). These arrest points serve to provide the cells more time to repair DNA damage before DNA replication and mitosis (ibid). The players involved in control of these arrest points differ. For example, the presence of damaged DNA in G1 phase prevents entry into S-phase (Maity et al. 1994). This arrest may be effected by inhibitory phosphorylation of G1 cyclin dependent kinases such as Cdk4 after irradiation (Terada et al. 1995). The G2 checkpoint in response to DNA damage relies on the inactivation or prevention of Cdc2 kinase activity to prevent progression into mitosis (Elledge, 1996; Nasmyth, 1996; Rhind et al. 1997). In mammalian cells, arrest at G2 is associated with decreased activity of Cdc2 kinase, and phosphorylation of the inhibitory sites of the Cdc2 protein (Kharbanda et al. 1994a;

Herzinger et al. 1995; Paules et al. 1995). Relief of the inhibitory phosphorylation is important for progression into mitosis. Mitosis is dependent on completion of DNA synthesis in *Xenopus* egg free extracts (Dasso and Newport, 1990). This block in mitosis results in the tyrosine phosphorylation of Cdc2 kinase that can be overcome by Cdc25 phosphatase or addition of either caffeine or the phosphatase inhibitor okadaic acid (Kumagai and Dunphy, 1991; Smythe and Newport, 1992). These treatments result in reduced tyrosine phosphorylation of Cdc2 kinase (ibid).

Recent work has suggested that control over Cdc25C has a central role in the response to DNA damage at G2. After DNA damage in yeast, inhibitory phosphorylation of Cdc2 on tyrosine 15 occurs, involving activation of Wee1/Mik1, inactivation of Cdc25C and arrest in G2/M (Rhind et al. 1997). This G2/M arrest point in yeast is mediated by the Chk1 kinase, which is essential for this arrest (O'Connell et al. 1997; Walworth et al. 1993; Al-Khodairy et al. 1994; Walworth and Bernards, 1996). Human Cdc25C becomes phosphorylated on Ser216 after DNA damage, which is an inactivating phosphorylation (Rhind et al. 1997; Sanchez et al. 1997; Peng et al. 1997). Both yeast and human Cdc25C are phosphorylated by the Chk1 kinase *in vitro* and *in vivo* (Furnari et al. 1997; Sanchez et al. 1997; Peng et al. 1997). This phosphorylation event is required for DNA damage induced arrest (ibid). Further, human cells expressing a non-phosphorylatable Ala216 mutant are defective for G2/M DNA arrest after DNA damage (Peng et al. 1997). Together these results suggest phosphorylation at Ser-216 on Cdc25 by Chk1 kinase is required for DNA damage induced growth arrest at G2 . Interestingly, Chk1 can also phosphorylate and activate Wee1 kinase, providing a second possible mechanism for Cdc2 inactivation (O'Connell et al. 1997).

Phosphorylation of Ser-216 permits binding of members of the 14-3-3 protein family (Peng et al. 1997). This also inhibits Cdc25 activity, possibly by sequestering Cdc25C and preventing it from activating Cdc2 (ibid).

It should be noted that the DNA replication checkpoint may not always require phosphorylation of tyrosine 15 on Cdc2, and that control over Cdc2 activity may involve at least two separate mechanisms. For example, inhibition of Cdc2/Cyclin B activity was not dependent on altered phosphorylation of any of the regulatory residues (Thr 14, Tyr 15 and Thr 161) in Xenopus egg extracts blocked in DNA synthesis (Kumagai and Dunphy, 1995). Instead, the results suggested the presence of an Cdc2/Cyclin B inhibitor. This inhibitor could be competitively removed by catalytically inactive Cdc2/Cyclin B (ibid). Evidence for a interphase inhibitor was also found in a *Xenopus* cell extract system using a non-phosphorylatable (threonine 14 changed to alanine, tyrosine 15 changed to phenylalanine) mutant of Cdc2, whose activity was still suppressed (Lee and Kirschner, 1996a). A membrane associated Cdc2 inhibitor has been biochemically characterized from *Xenopus* extracts, although its function remains unknown (Lee and Kirschner, 1996b).

Similar to the *Xenopus* cell extract experiments, expression of phosphorylation site mutant (Cdc2AF) in HeLa cells does not induce premature mitosis in cells arrested in early S phase (Jin et al. 1996). Nonetheless, 25% of Cdc25AF expressing cells further into S phase did display signs of premature mitosis (ibid). In addition, these cells exhibit a reduced mitotic delay after X-irradiation (ibid). This suggests there may be at least a two stage control over Cdc2 activity in both mitosis and DNA damage response. Cdc2 may be initially controlled by one or more inhibitor proteins early in S phase, with negative

phosphorylation coming into play later in the cell cycle.

Two examples for a Cdc2 inhibitor can be found in yeast. Rum1 in fission yeast inhibits G1 and G2 progression (Labib and Moreno, 1996) by inhibiting Cig2/Cdc2 complex activity, and prevents premature DNA synthesis and premature mitosis in G1 (Moreno and Nurse, 1994; Correa-Bordes and Nurse, 1995). Inhibition of Cdc13/Cdc2 kinase activity by elevated Rum1 expression induces multiple rounds of DNA synthesis without mitosis (Correa-Bordes and Nurse, 1995). *S. cerevisiae* has the Sic1 protein which functions after cell cycle initiation to prevent premature S phase (DNA replication) (Mendenhall, 1993; Donovan et al. 1994; Nugroho and Mendenhall, 1994; Schwob et al. 1994). Together, this suggests that control over Cdc2/cyclin B activity is important to prevent multiple rounds of DNA synthesis and mitosis.

One other point of control in the cell cycle is at the level of spindle formation during mitosis. Proper chromosome segregation at mitosis requires correct spindle assembly (Rieder et al. 1994). Several candidate genes for spindle control in yeast have been identified, including the mitotic arrest deficient (Mad) 1-3 genes (Guenette et al. 1995; Roberts et al. 1994) and the budding by binomial (Bub)1-3 genes (Roberts et al. 1994; Wang and Burke, 1995; Guenette et al. 1995). In vertebrates, Erk2 kinase, a member of the MAP kinase family, is activated by spindle depolarization, inducing arrest at mitosis in *Xenopus* eggs and embryos (Minshull et al. 1994). Dephosphorylation of Erk2 by a specific phosphatase inactivates it and releases the cells from growth arrest (ibid). P53 may have a role in spindle checkpoint control via control of the centrosome duplication, as will be discussed (Fukasawa et al. 1996).

2.3 p53 control over G1/S progression:

p53 protein is stabilized in response to DNA damage and other stresses, inducing growth arrest (Kastan et al. 1992; Lu and Lane, 1993). This effect on G1 progression occurs just before the restriction, or R point (Lin et al. 1992). It is fairly well established that p53 exerts its control over the cell cycle at this point by transcriptional control over the $p21^{waf1/cip1}$ gene (reviewed in (Morgan and Kastan, 1997), please also see Figure 4). $P21^{waf1/cip1}$ is a Cyclin dependent kinase (cdk) inhibitor (Gu et al. 1993; Harper et al. 1993; Xiong et al. 1993) which is expressed at high levels in senescent cells (Noda et al. 1994). The $p21^{waf1/cip1}$ gene was first identified as a p53 transcriptional target in 1993 (el Deiry et al. 1993). $P21^{waf1/cip1}$ is most effective at inhibiting the G1/S cdks, including Cdk2, Cdk3, Cdk4 and Cdk6 kinases, but works very poorly on the G2 kinase Cdc2 (Cdk1) (Harper et al. 1995).

P53 also indirectly regulates the phosphorylation of Retinoblastoma (Rb) protein, keeping the protein in a hypophosphorylated state (Demers et al. 1994; Slebos et al. 1994). In its hypophosphorylated form, Rb protein binds and inhibits members of the E2F transcription factor family, preventing entry into S phase (Nevins, 1992; Helin et al. 1993; Lees et al. 1993). Phosphorylation of Rb protein by G1 kinases (Cyclin D-cdk4 or Cyclin E-cdk2) releases E2F and allows progression into S phase (for review, see (Beijersbergen and Bernards, 1996; Levine, 1997; Muller, 1995; Sherr, 1996)). The timing of p53 induced hypophosphorylation of Rb is important. A recent report demonstrated that even though p53 could still mediate accumulation of hypophosphorylated Rb in human fibroblasts past the

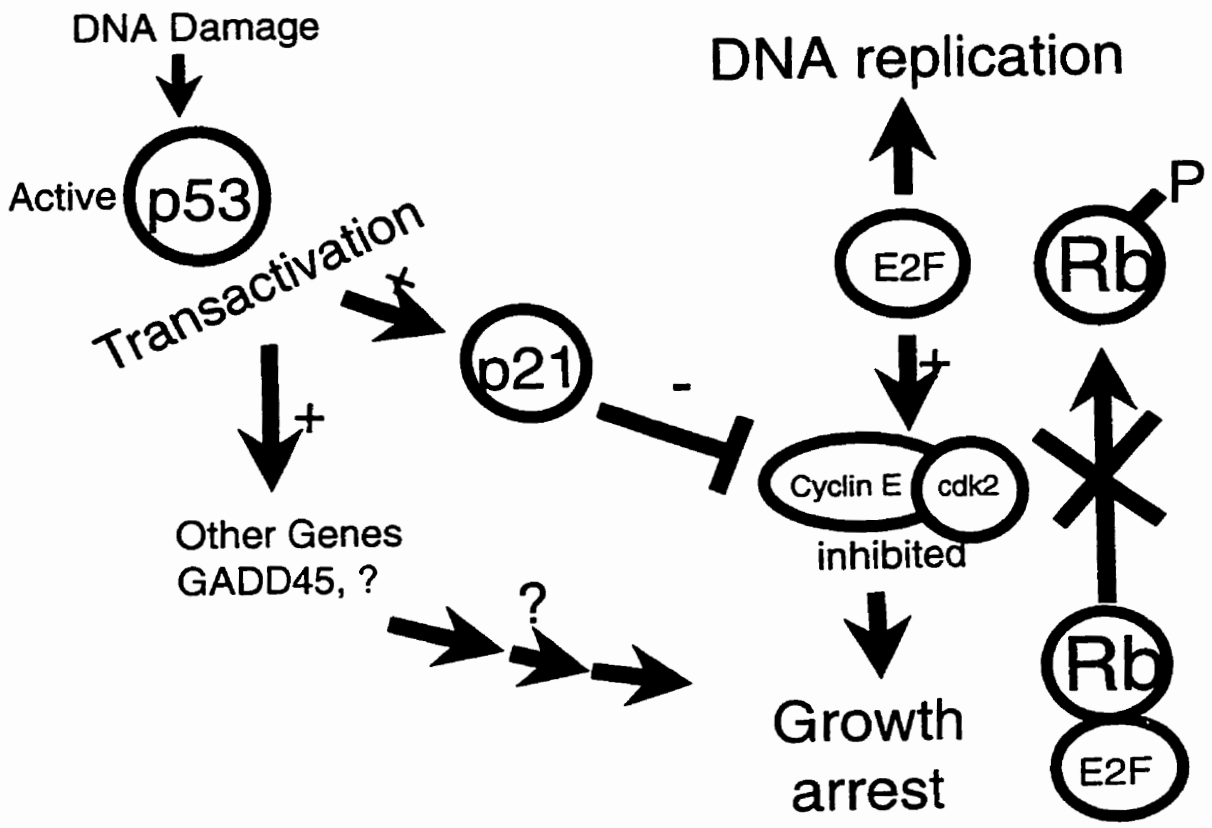

Figure 4: p53 control over G1 progression. p53 protein, transcriptionally activated by stimuli such as DNA damage. The protein then binds to the $p21^{waf1/cip1}$ promoter, inducing its expression. $p21^{waf1/cip1}$ protein binds to and inhibits the activity of Cyclin-Cdk complexes necessary for the G1 to S transistion, arresting the cells in late G1. This arrest may also involve transactivation of additional genes such as Gadd45. Adapted from Ko and Prives, 1996, Macleod et al., 1996, and Levine, 1997.

restriction point (late G1), it failed to prevent these cells from entering S phase (Linke et al. 1997b). This implies that once cells have passed the restriction checkpoint they may be irreversibly committed to enter S phase, regardless of the actions of either p53 or Rb (ibid).

Notwithstanding the above, induction of p21 $^{waf1/cip1}$ may not be the entire story for p53 induced G1/S arrest. Studies on p21 $^{waf1/cip1}$ deficient mice show only a partial defect in G1 arrest after DNA damage or nucleotide pool perturbations (Deng et al. 1995; Brugarolas et al. 1995), suggesting other components are required for complete growth arrest. P21$^{waf1/cip1}$ mice did not show defects in apoptotic control or spindle checkpoint control (ibid). These mice did not show an increased tumour incidence suggesting that p21$^{waf1/cip1}$ is not important for p53's tumour suppressor functions (ibid).

This means that other gene/proteins are also important for p53 action at G1. One potential target may be the GADD45 gene, which is also a transcriptional target of p53 (Kastan et al. 1992). GADD45 is a DNA damage inducible gene that can cause growth arrest when overexpressed in cells (Zhan et al. 1994b). Cdc2 protein/mRNA have been shown recently to be downregulated in a p53 dependent manner after irradiation in G0-G1 synchronized human fibroblasts (Azzam et al. 1997). This corresponded with delay in initiating DNA synthesis (ibid). P53 can also induce the expression of another cell cycle protein, Cyclin G (Okamoto and Beach, 1994; Zauberman et al. 1995). Cyclin G protein has been recently shown to complex with the B' regulatory subunits of protein phosphatase 2A (PP2A) (Okamoto et al. 1996). PP2A regulates many processes including signal transduction, cell cycle, transcription and development (reviewed in (Mumby and Walter, 1993)). However, Cyclin G overexpression enhances cell cycle progression and increases

cisplatin sensitivity (Skotzko et al. 1995; Smith et al. 1997), making its role in p53 G1 arrest uncertain.

2.4 Non-Transcriptional Control Over Cell Cycle by p53

The above mentioned arrest strategies rely upon the transcriptional regulatory abilities of p53. P53 also possesses a non-transcriptional control over cell cycle through its proline rich region (Walker and Levine, 1996). The poly-proline (PP) region of p53, comprised of amino acids 58-93 (ProXXPro amino acid repeats) can bind to SH3 homology domain 3 (SH3) proteins, which are involved in tyrosine kinase signal transduction (ibid). Deletion of this region does not affect transactivation by p53 (ibid). Transfection of a PP region mutant into Saos-2 cell lines reduced colony formation by 2 fold, compared to 10 fold for wild-type (ibid). The PP region of p53 is required for growth arrest induced by the Gas1 gene (Ruaro et al. 1997). Gas1 requires transcription-independent functions of p53 to induce growth arrest when etopically expressed in cells (Del Sal et al. 1995).

2.5 p53 in Oncogenic pathways and Senescence

p53 is also part of a negative growth control pathway, resulting in arrest at both G1 and G2/M, induced by overexpression of the Ras oncogene (Hirakawa and Ruley, 1988; Hicks et al. 1991). This is one example where loss of p53 is required to relieve growth arrest caused by oncogene overexpression. Ras acts near the top of a serine/threonine protein kinase cascade consisting of Raf, MEK, MAPK. Ras is a molecular switch for reentry into

the cell cycle at G0/G1, by transmitting extracellular mitogenic stimuli (for review, see Downward, 1997). Ras also functions in late G1 to downregulate p27kip1 and allow passage through the restriction point (Takuwa and Takuwa, 1997).

The suggestion that p53 may function as part of a negative growth pathway involving Ras came from several sources. Transfection of Ras into mouse primary prostate cells always results in selection for p53 mutations (Lu et al. 1992), however cotransfection of Myc and Ras induced carcinomas that expressed elevated levels of wild type p53 protein and increased levels of apoptosis (ibid). Elevated Ras expression in the REF52 (rat embryo fibroblast) cell line induces a block at both G1/S and G2/M that can be overcome by SV40 large T antigen expression (Franza et al. 1986; Hirakawa and Ruley, 1988). Similarily, large T antigen can overcome G1/S and G2/M arrest induced in Schwann cells transfected with Ras (Ridley et al. 1988). This arrest can be alieviated by inactivation of p53 function through transfection of a dominant negative mutant p53, which allows for elevated Ras expression and increased tumourigenicity in REF52 cells (Hicks et al. 1991).

Activation of Ras and loss of functional p53 activity may be important for the avoidance of senescence. Ras oncogene-induced growth arrest is associated with premature senescence in primary fibroblasts (Serrano et al. 1997), which can be overcome by inactivation of either p53 or p16 in rodent cells. Neither p53 nor p16 were sufficient to overcome Ras growth arrest in human cells, but E1A coexpression was sufficient (ibid). This Ras induced growth arrest is similar to the premature senescence seen in normal diploid fibroblasts induced by γ irradiation that is dependent on p53 (Di Leonardo et al. 1994; Linke et al. 1997a). Addition of mutant p53 to near-senescent fibroblasts also allows an extension

of their proliferative lifespan (Bond et al. 1994). Senescence is presumably due to unrepaired DNA strand breaks causing p53 stabilization, critical shortening of telomeres and induction of p53 transcription-dependent factors like $p21^{waf1/cip1}$ (ibid; Bond et al. 1996; Vaziri et al., 1997). Induction of $p21^{waf1/cip1}$ has been previously associated with senescent cells (Noda et al. 1994). Reintroduction of wild type p53 into EJ bladder carcinoma cells (lacking functional p53) resulted in $p21^{waf1/cip1}$ induction and the repression of mitotic Cyclin/cdk complexes followed by the induction of senescence (Sugrue et al. 1997). This arrest became permanent after 48-72 hours, demonstrating that p53 overexpression can activate senescence in tumour cells (ibid). Telomere erosion seen in normal aging and associated with senescence may trigger p53 dependent growth arrest (Bond et al. 1994; Wynford-Thomas et al. 1995). This phenomenon may not directly induce senescence, but be part of permanent growth arrest, since p53 arrest can be reversed when induced by a decrease in ribonucleotide pools or by using an inducible system (Agarwal et al. 1995; Linke et al. 1996). Thus, constitutive Ras expression, or DNA strand breaks left unrepaired may in concert with p53 be critical events in permanent arrest.

At which point p53 works downstream in the Ras pathway is uncertain. Overexpression of other members of the MAP kinase pathway such as Ras, Raf, MEK, or the Mos oncogene induces growth arrest and apoptosis in fibroblasts (Fukasawa and Vande Woude, 1997), which is greatly reduced in p53 null fibroblasts, suggesting that p53's action occurs after these steps. Further, $p21^{waf1/cip1}$ mediates growth arrest induced by Raf oncogene overexpression in primary rat Schwann cells, and can be overcome by mutant p53 or large T antigen (Lloyd et al. 1997). P53 might directly influence Ras pathway function through

A28-RGS14, a p53 inducible gene(Buckbinder et al. 1997). A28-RGS14 is a novel factor which can inhibit G protein coupled mitogenic signal transduction and activation of the MAPK pathway (ibid). How p53 is activated in response to Ras stimulation remains to be determined. Overexpression of Ras/MAPK pathway members can lead to genomic instability and apoptosis (Denko et al. 1994; Denko et al. 1995; Fukasawa and Vande Woude, 1997), which might also indirectly induce p53 action. p53 is subject to phosphorylation by MAP kinase (Milne et al. 1994), providing a possible direct mechanism for p53 response to Ras. In summary, oncogene overexpression by genes such as Ras may have consequences which lead to p53 activation, such as the formation of strand breaks, DNA damage and genomic instability.

2.6 p53 and Mitotic Checkpoints

Another area of p53 control over cell cycle is its function as part of mitotic spindle checkpoint control. Human Papilloma virus (HPV) 31 E2 protein can abrogate a mitotic checkpoint, E2 expression in squamous cell carcinoma cell line SCC-13 induced accumulation in S phase where the cells underwent multiple rounds of replication without mitosis. The level of p53 protein was reduced suggesting a checkpoint controlled by p53 (Frattini et al. 1997). In p53 deficient cells disruption of the mitotic spindle complex with drugs that normally arrest at G2/M, allows multiple rounds of DNA synthesis without cytokinesis (Cross et al. 1995). This p53 checkpoint function may operate independently of $p21^{waf1/cip1}$, as $p21^{waf1/cip1}$ deficient mice did not show any defect in spindle or G2 checkpoint control (Brugarolas et al. 1995; Deng et al. 1995).

This mitotic arrest may be related to p53's control over centrosome duplication. Fibroblasts from p53 deficient mice show multiple copies of centrosomes that result in abnormal segregation of chromosomes (Fukasawa et al. 1996). Control over this spindle fibre checkpoint may be important in preventing endoreduplication, and the production of aneuploid (including polyploid) cells is often observed in p53 deficient cell populations (Harvey et al. 1993; Tsukada et al. 1993; Galipeau et al. 1996; Lee and Kirschner, 1996a). 14-3-3σ has recently been identified as a potential p53-regulated inhibitor of mitosis (to be discussed later; Hermeking et al. 1997).

2.7 P53 and G2 DNA Damage Checkpoints

Whether p53 functions as part of the G2/M checkpoint control in response to unreplicated or damaged DNA is unknown. P53 deficient cells still show a G2/M arrest after irradiation (Kuerbitz et al. 1992; Kastan et al. 1991). However, more than one cell cycle checkpoint may be in place at G2/M to regulate Cdc2 kinase and induce G2/M arrest, with or without p53 action. Activation of the Lyn tyrosine kinase induces a G2/M arrest in response to irradiation in haematopoietic cells (Kharbanda et al. 1994b; Kharbanda et al. 1996; Uckun et al. 1996). Ionizing radiation (IR) induces rapid activation of the Lyn kinase, which in turn can bind to Cdc2 kinase and results in inhibitory phosphorylation at the Tyrosine 15 residue of Cdc2 kinase (Kharbanda et al. 1994b; Kharbanda et al. 1996; Uckun et al. 1996). Further, IR fails to cause tyrosine phosphorylation of Cdc2 kinase at G2 arrest in Lyn -/- B-cell precursors (Uckun et al. 1996). To date no evidence has been presented to show p53 works on this pathway.

c-Abl tyrosine kinase is also activated in response to DNA damaging agents such as irradiation (Kharbanda et al. 1995), and the BCR-ABL protein chimera can induce a G2/M growth arrest when expressed at elevated levels in haematopoietic cells (Bedi et al. 1995). This protects the cells from radiation and chemotherapeutic induced apoptosis (ibid). Overexpression of c-Abl also induces growth arrest at G1 through binding to p53 protein (Sawyers et al. 1994; Goga et al. 1995). Binding of active c-Abl to p53 is also required for the induction of growth arrest in response to genotoxic drugs or irradiation and is associated with down regulation of cdk activity, but does not require transactivation of the $p21^{waf1/cip1}$ gene (Yuan et al. 1996; Yuan et al. 1996). c-Abl and p53 also associate in response to genotoxic drugs, such as antimetabolite 1-beta-D-arabinofuranosylcytosine (ara-C) and the alkylating agent methyl methanesulfonate (MMS) (Yuan et al. 1996). This response is associated with increased expression of $p21^{waf1/cip1}$ (cdk2 inhibitor), with downregulation of Cdk2 only seen in cells possessing wildtype c-Abl and p53 (ibid). Thus, several complementary pathways exist for arrest at G2 in response to genotoxic stress, which may or may not require p53 function. The role p53 has in G2 control is the focus of this study.

2.8 p53 and G2 arrest.

Wild-type p53 is responsible for an ionizing radiation-induced G1 checkpoint control, but is not required for the G2 arrest response (Kastan et al. 1991; Kuerbitz et al. 1992). As outlined above, however, multiple mechanisms may be in place to ensure arrest at G2, some of which may involve p53 function. Previously, it had been noted that Ras overexpression induces G2 arrest, which can be overcome by the addition of dominant negative p53 mutant

(Hicks et al. 1991). This was suggestive of a G2/M arrest point mediated by p53 expression. Thus, this project was devised to determine what role p53 had in the progression through G2/M. Since wild type overexpression is not generally tolerated by most cell types, a system based on the temperature sensitive val135 p53 mutant originally identified in 1988 (Finlay et al. 1988) was established. This mutant, which encodes valine instead of alanine at position 135, retains a mutant conformation at 37°C but at lower (32°C to 35°C) temperatures regains wild type conformation and function (Michalovitz et al. 1990; Milner and Medcalf, 1990). Val135 at the nonpermissive temperature remains in the cytoplasm, and lacks DNA binding activity (Martinez et al. 1997a). At the permissive temperature, the protein moves into the nucleus and becomes hyperphosphorylated on the N-terminus (phosphorylation at the C-terminus happens in both the nucleus and cytoplasm). This can increase sequence-specific binding to DNA and upregulate Mdm2 and $p21^{waf1/cip1}$ (Martinez et al. 1997a). A plasmid encoding for this mutant was placed in the REF52 (rat embryo fibroblast) cell line. This resulted in stable cell lines that could express wild type p53 on demand. Using this system I asked the question whether 1) p53 could induce an arrest point at G2/M, and 2) if so, what were the possible mechanisms behind this block. The following summarizes the results of this investigation.

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Evidence for a Second Cell Cycle Block at G2/M by p53

PREFACE

The following chapter is the full paper format of a manuscript published in the journal Oncogene: Nancy Stewart, Geoffrey G. Hicks, Frixos Paraskevas and Michael Mowat. 1995. Evidence for a Second Cell Cycle Block at G2/M by p53. Oncogene 10: 109-115.

This paper was one of the first to provide evidence that as well as the initially characterized block at G1, p53 was able to arrest cells at a second point in G2/M.

Evidence for a Second Cell Cycle Block at G2/M by p53

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ABSTRACT

Wild type p53 can induce cell cycle arrest at specific points in the cell cycle, in particular G1/S, an ability lost by most p53 mutants. We have previously reported that p53 mutant genes can rescue REF52 cells from *ras*-induced growth arrest and that over expression of wild type p53 inhibits cell growth in these cells. In this paper we examined whether p53 can also induce cell cycle arrest at the G2/M boundary of the cell cycle. To accomplish this we used the REF52 cell line and the temperature sensitive p53val^{135} mutant allele. Cells were enriched in the late G1 and early S phases before the temperature shift. REF52 cells expressing mutant-p53val^{135} alone or with an activated *H-ras* gene arrest primarily at the G1/S and G2/M parts of the cell cycle at the restrictive temperature, as determined by flow cytometry analysis. These results suggest that the anti-proliferative activity of p53 may be involved in regulation of the cell cycle at the G2/M restriction point as well as transit through G1/S and initiation of DNA synthesis.

INTRODUCTION

It is now clear that wild-type p53 (wtp53) is a tumor suppressor gene characterized by its tumor-suppressing (Eliyahu *et al.*,1989; Finlay *et al.*,1989; Hinds *et al.*,1989), and anti-proliferative activity (Baker *et al.*,1990; Mercer *et al.*,1990a; Johnson *et al.*,1991; for a review, see: Levine, 1993; Rotter *et al.*,1993). Although the exact mechanism of its action as a negative growth regulator remains unclear, it is likely related to its recent demonstration of DNA specific binding (Kern *et al.*,1991; Funk *et al.*,1992; Foord *et al.*,1993; Friedman *et al.*,1993), transcriptional activation (Fields and Jang, 1990; O'Rourke *et al.*,1990; Raycroft *et al.*,1990), transcriptional repression (Ginsberg *et al.*,1991a; Shiio *et al.*,1992; Subler *et al.*,1992; Agoff *et al.*,1993), and binding of transcription factors TFIID and TBP (Seto *et al.*,1992; Chen *et al.*,1993; Liu *et al.*,1993; Ragimov *et al.*,1993; Truant *et al.*,1993).

Alteration of p53 appears to be the single most common genetic lesions in human cancer (Hollstein *et al.*,1991; Levine, 1993). Loss of wtp53 has been observed by allelic inactivation through deletion or rearrangement (Wolf *et al.*,1984a; Mowat *et al.*,1985; Masuda *et al.*,1987; Baker *et al.*,1989; Takahashi *et al.*,1989). More often, p53 alterations are missense mutations found in highly conserved coding regions of the protein reviewed in (Lane and Benchimol, 1990; Levine, 1993). This generally results in a common conformational change of the protein that is detectable by differences in immunoreactivity such as the loss of an epitope for the monoclonal antibody PAb246 (Milner and Cook, 1986; Yewdell *et al.*,1986; Sturzbecher *et al.*,1987; Finlay *et al.*,1988) and expression of a new epitope for the monoclonal antibody PAb240 (Gannon *et al.*,1990). The conformational

change is associated with over expression of a more stable mutant p53 protein, the ability to oligomerize with both itself and wild type p53 proteins, and to alter the conformation of newly translated wtp53 protein, all affecting wtp53 function in a dominant negative manner (Eliyahu *et al.*,1988; Kraiss *et al.*,1988; Rovinski and Benchimol, 1988; Gannon *et al.*,1990; Milner and Medcalf, 1991; Milner *et al.*,1991; Slingerland *et al.*,1993; Unger *et al.*,1993). Such mutations are frequently oncogenic with immortalizing (Jenkins *et al.*,1984; Rovinski and Benchimol, 1988), transforming (Jenkins *et al.*,1984; Parada *et al.*,1984; Eliyahu *et al.*,1988), and tumor enhancing activity (Wolf *et al.*,1984b; Eliyahu *et al.*,1985).

The observation that a p53val^{135} mutant is temperature sensitive for the wild-type or mutant conformation (Michalovitz *et al.*,1990) has permitted conformation-dependent activities of p53 to be tested directly. When the p53val^{135} protein is switched to the wild-type conformation, it can conditionally suppress transformation and inhibit proliferation of transfected rat embryo fibroblasts (Michalovitz *et al.*,1990; Martinez *et al.*,1991), and murine erythroleukemic cells lacking p53 (Johnson *et al.*,1993; Ryan *et al.*,1993) as well as induce apoptosis in leukemic cells (Yonish Rouach *et al.*,1991; Johnson *et al.*,1993; Ryan *et al.*,1993). Over expression of wtp53 in fibroblasts results in a reversible growth arrest primarily in late G1, near the restriction point (Lin *et al.*,1992). This pattern of growth arrest in response to wtp53 over expression was also demonstrated using an inducible p53 construct (Mercer *et al.*,1990b). Further studies have shown that negative regulation of the cell cycle by p53 is wild-type conformation dependent and that this is integrally associated with cellular localization to the nucleus during G1 (Ginsberg *et al.*,1991b; Gannon and Lane, 1991; Martinez *et al.*,1991). These reports suggest that p53's conformation and cellular

localizations are the normal regulators of wtp53 during the cell cycle (Cook and Milner, 1990; Milner and Watson, 1990; Shaulsky *et al.*, 1991).

Negative growth regulation in the rat embryo fibroblast line REF52 can be induced by the T24/EJ *ras* oncogene (Franza *et al.*, 1986). Expression of this transfected gene to levels higher than 10 to 30% of the endogenous rat p21 level results in growth arrest and subsequent lethality of the cell (Franza *et al.*, 1986). This arrest can be rescued by co-transfer of either Adenovirus 5 E1A or simian virus 40 (SV40) large T antigen genes (Franza *et al.*, 1986; Hirakawa and Ruley, 1988). In a previous publication, we have shown that p53 could also rescue REF52 cells from *ras*-induced growth arrest. Introduction of dominant negative mutant p53 alleles was able to abrogate the negative growth regulation induced by *ras*, while normal endogenous or introduced exogenous wtp53 alleles could not (Hicks *et al.*, 1991). These results imply that p53 is part of a negative growth control pathway induced by *ras* oncogene over expression. Therefore part of the basis for co-operation between oncogenes and tumor suppressor gene inactivation in transformation is to overcome a negative growth control pathway induced by elevated oncogene expression (for review see Ruley, 1990). Hirakawa and Ruley (1988) showed that REF52 cells, carrying a temperature sensitive large T antigen and *ras* oncogene, would growth arrest primarily at G2/M of the cell cycle at the non permissive temperature. Since previous studies have suggested that p53 primarily block cells at the G1/S restriction point, this prompted us to reevaluate cell cycle control of p53 in REF52 cells using the temperature sensitive p53val^{135} allele. We report the presence of a second p53 induced growth arrest point at G2/M.

RESULTS

p53val^{135} temperature sensitive inhibition of cell growth

To study the role of p53 in cell cycle control of the REF52 cell line we used cells expressing various mutant p53 genes including the temperature sensitive allele p53val^{135}. These cell lines were tested for their growth rates and viability at the permissive and restrictive temperatures. All cell lines studied grew exponentially under non-restrictive incubation conditions (39°C), and with similar rates. When the temperature was shifted to 32.5°C, the parental REF52 cell line maintained an exponential rate of growth but with an increased doubling time (Fig. 1). Cells transformed by the non temperature-sensitive p53pro^{193} allele plus *ras* (Fig. 1) showed a similar pattern. In the cell line LTR/4, where *ras*-induced growth arrest has been rescued by the temperature-sensitive p53val^{135}, there is a dramatic drop in the growth potential (Fig. 1). Although most of the cells appear to have growth arrested, there remains a sub-population of cells that can continue cell division at a greatly reduced rate. This likely reflects a "leaky" nature of the temperature-sensitive wild-type-p53val^{135} conformation. In one of the six LTR/4 experiments, the growth curve declined steadily from the original 4×10^4 cells. Finally, cell line LTR/6, which was transfected with only p53val^{135}, shows a growth arrest similar to the LTR/4 cells following the temperature shift.

p53 epitope expression.

Changes in the expression of wtp53 conformation or mutant p53 conformation can generally be detected by the monoclonal antibody Pab246 or Pab240, respectively. We wished to use the expression of these epitopes to both confirm a temperature-dependent conformational change in p53val^{135}, and to follow the dynamics of the epitope expression immediately following the temperature-shift, by densitometric analysis. The expression of each epitope, taken from the same metabolically labelled cell extract, is expressed as a percentage of initial p53 expression (designated 100%). One should bear in mind that p53val^{135} transfected REF52 cells express ≈15 fold higher levels of p53 than does the normal parental line (Hicks *et al.*, 1991).

At 24 hrs post-temperature-shift, LTR/4 (p53val^{135} plus *ras*) cells showed a drop in the relative Pab240 expression of 32.3%. A initial decline at 6 hours followed by a modest recovery (10.1%) is observed over a 24 hr period (Fig. 2A). The relative PAb246 epitope expression rose by a difference of 74.1% (Fig. 2B). LTR/6 (p53val^{135}) showed similar change, but with more dramatic differences. Relative PAb240 expression decreased to 37.6%, and relative PAb246 expression rose to 204.9%. These changes were not continuously gradual in the LTR/6 cell line. The cells showed a slight increase in the relative expression of PAb240 epitope at six hr before declining to the lower 24 hr level. This increase was coincident with a dramatic drop in the relative PAb246 epitope expression in the LTR/6 cells at six hours before rising to levels above the initial ones.

DNA and cell cycle analysis of growth arrest.

A previous study by Hirakawa and Ruley (1988) using a temperature sensitive SV40 large T antigen and *ras* transformed REF52 cell line showed that elevated expression of the *ras* oncogene at the restrictive temperature for large T antigen results in a cell cycle block at both G1/S and G2/M parts of the cell cycle. If p53 is part of this *ras* induced negative growth control pathway as our previous results suggest (Hicks *et al.*, 1991), then p53 should also induce a double block in the cell cycle. This concept had also been suggested by the results of our initial cell cycle analysis. In these experiments, asynchronous cell populations of the two cell lines, LTR4 and LTR6, were growth arrested by incubation at the nonpermissive temperature for 3 days. Their cell cycle distribution as determined by propidium iodide staining and DNA flow cytometry is shown in Figure 3. DNA fluorescence data were cell cycle analyzed using Multicycle™ software (Phoenix Flow Systems), which allows for the removal of G1 doublets during analysis. As expected, a normal distribution was observed at the permissive temperature (39°C) for both the LTR4 (p53val^{135} + *ras*) and LTR6 (p53val^{135}) cell lines. A normal distribution was also observed with the parental cell line at both 32°C and 39°C. Interestingly, following the temperature shift, although the LTR4 and LTR6 cell lines arrested primarily in G1 with the loss of S phase, we did observe a significant percentage of cells arrested in G2 (Figure 3).

Since it has been firmly established that p53 can arrest cells at the G1/S restriction point we wanted to directly test whether p53 could also arrest cells at the G2/M part of the cell cycle. To address this, we chose to track the progression of cells in S phase after the shift to non-permissive temperature. LTR4 and LTR6 cells arrested by serum starvation were

released and allowed to progress for 18 hrs before labelling for two hours with bromodeoxyuridine. After Budr labelling the cells were shifted to 32°C or left at 39°C and collected over time. The data was analyzed using EPICS Cytologic DNA Package (Version 2.1), which allows for the subtraction of unlabelled cells before cell cycle quantitation, and is expressed on a linear scale. The results from these experiments are shown in Figure 4. In both cases, labelled cells at the permissive temperature progressed through G2/M, and continued to cycle for the duration of the experiment (Figures 4a and b). In contrast, labelled cells at the non-permissive temperature arrested at G1 and G2/M within 24 hours of the temperature shift. Few S phase cells were present after this time. G2/M arrested populations were still present 90 hours after labelling (data not shown). To verify that the cells at 32°C had enough time to progress through G2/M, the parental cell line REF52 was labelled and placed at 32°C (Figure 4c). These results confirmed that these cells passed through G2/M within 12-18 hours after labelling, and continued to cycle normally for the duration of the experiment.

Wild type p53 overexpression is also associated with apoptosis. We occasionally see cells with a DNA content less than 2N, which can suggest cells undergoing apoptosis (Darzynkiewicz *et al.*, 1992). However, we found no evidence of typical DNA ladder profile or nuclear condensation and fragmentation indicative of apoptosis in these cells at the restrictive temperature either in the presence or absence of serum (data not shown).

DISCUSSION

In this report we show that REF52 cells transfected with a temperature-sensitive (*ts*-) p53val^{135} with or without the T24/EJ *ras* oncogene show a conditional block at two points in the cell cycle; G1/S and G2/M. This pattern is similar to the growth arrest observed in *ras* transformed REF52 cells conditionally rescued by a *ts*-SV40 large T antigen (Hirakawa and Ruley, 1988). These results further support the notion that p53 is on a negative growth control pathway induced by over expression of the *ras* oncogene as revealed in the REF52 cell line. It should be noted that Schwann cells are also sensitive to ras oncogene over expression that can be rescued by SV40 large T antigen expression (Ridley *et al.*,1988). Further, our results suggest p53 may be involved in regulation of the cell cycle at more points than G1/S, as previous results have suggested (Mercer *et al.*,1990b; Martinez *et al.*,1991). Michalovitz *et al.* (1990) have found growth arrest induced by wtp53 can be seen at other parts of the cell cycle to a lesser degree, most notably at G2/M in rat embryo fibroblasts cells. The study of Martinez et al. (1991) who also used cell synchronized in S phase prior to the temperature shift shows a similar modest peak of G2/M cells, however the authors did not report the fate of this peak past 24 hours. Since it takes approximately 24 h to see a peak in the Pab246 epitope associated with wild type p53 (Figure 2), some cells in S phase or G2/M could still progress until sufficient levels of wild type p53 are achieved to produce a block. In fact we see some of the cells labelled in S phase progressing to the G1 block as well as to the G2/M block (Figure 4).

This leads to the question why other studies failed to see the G2/M block by p53. The G2/M transition is the shortest part of the cell cycle so the number of cells in G2/M at

any one time is small (Baserga, 1985). Another possibility is that G2/M blocked cells are undergoing apoptosis resulting in loss of cells with a 4N DNA content. Friend erythroleukemic cell lines transfected with the same p53val^{135} allele undergo apoptosis and a cell cycle block when shifted to the non-permissive temperature (Johnson et al., 1993; Ryan et al., 1993). Johnson et al. (1993) found that erythropoietin prevented p53 induced apoptosis but not the cell cycle block. Interestingly, under these conditions the G1 and G2/M DNA peaks were increased. In contrast, blocked cells not receiving erythropoietin lost the G2/M DNA peak with time and showed an increase of cells with a DNA content less than 2N (apoptotic cells). We found no evidence for apoptosis in REF52 cells when wild type p53 is over expressed with or without the *ras* oncogene. This may indicate that over expression or stabilization of p53 alone is insufficient to induce apoptosis in REF52 cells.

Recently, a p53 splice variant (p53as) that is preferentially expressed at the G2 part of the cell cycle has been described (Kulesz-Martin *et al.*, 1994). This may be related to the G2/M block we report. The p53 clone (p53val^{135}, Eliyahu *et al.*, 1985) we used should be able to produce p53as as it contains the neccessary 3' genomic sequences. Since some previous studies on p53 induced cell cycle arrest used p53 cDNA clones that would not contain this variant, this may explain why the G2/M arrest has not been as widely observed. The role of p53as in both cell cycle blocks will be the subject of future work.

A second potential mechanism for the G2/M block may be the CDK inhibitor p21 (also called WAF-1, Cip1, Pic1). p21 in normal cells complexes and down regulates the activity of cdk-cyclins (A,B1,D,E) (Gu *et al.*,1993; Harper *et al.*,1993; Xiong *et al.*,1993). p21 is known to be transactivated by wtp53 (El-Deiry *et al.*,1993). Expression of p21 also

increases (10 fold) after gamma irradiation, suggesting a role in inducing cell cycle arrest in response to DNA damage (Xiong *et al.*, 1993b). Further, in transformed and tumorigenic cell lines both expression of p21 and complex formation is severely decreased. Interestingly, expression and complex formation are also absent or greatly reduced in both p53 negative fibroblasts and cells obtained from Li-Fraumeni patients (Xiong *et al.*, 1993b). p21 is also induced in cells undergoing p53-associcated G1 arrest and apoptosis (Dulic *et al.*, 1994; El-Deiry *et al.*, 1994). Together these findings suggest that p53 activates p21 expression, consequently down regulating the action of the cell cycle kinases and thus inducing cycle arrest.

Whether p53 control of p21 expression is responsible for the G2/M block induced by p53 is not known at this time. Since the cdc2/cyclin B kinase is needed for progression through G2/M (Pines, 1991), this suggests that p53 may control expression of either p21 or another related cdc2 interacting protein active at the G2/M part of the cell cycle. In support of this hypothesis is a recent paper by Li et al. (1994), which reports that p21 can associate with cyclin B1/cdc2/PCNA complexes at the G2 phase of the cell cycle, and that the level of p21 mRNA increases as cells enter G2 and M phase. We are currently investigating whether p21 expression is involved in the G2/M arrest.

MATERIALS AND METHODS

Cells and Culture.

REF52 cells are an established rat embryo fibroblast line (Franza *et al.*, 1986) and were a gift from H. Earl Ruley. The following lines were derived in our laboratory and contain transfected genes that stably express the proteins shown in parentheses as follows: LTR/1 (p53val^{135} plus T24/EJ *ras*); LTR/4 (p53val^{135} plus T24/EJ *ras*); LTR/6 (p53val^{135}); R53/4 (p53pro^{193} plus T24/EJ *ras*) (Hicks *et al.*, 1991). p53val^{135} is wild-type p53 conformation at 32.5°C or a mutant p53 conformation at 39°C; it is encoded by plasmid pLTRp53cG6 (Eliyahu *et al.*, 1985). These cells were maintained at 39°C since we found that p53val^{135} allele to be partially wild type at 37°C. Low passage REF52 cells and all cell lines were maintained in Dulbecco's alpha minimum essential media supplemented with 10% fetal bovine serum in a 5% CO_2 atmosphere at 37°C, unless otherwise shown.

Cell Cycle Analysis.

Cells were seeded and incubated as indicated. Monolayers of cells were washed twice with cold phosphate buffered saline (PBS), trypsinized, and washed twice more with cold TSE (TSE is O.100 M Tris-HCl, O.O70 M NaCl, 0.005 M EDTA; adjusted to pH 7.5, and filter sterilized). Final cell pellets (1 to 2 × 10^6 cells) were resuspended into 750 µl cold TSE and fixed by drop-wise addition of 2.0 ml of 95% ethanol (-20°C) while gently vortexing, and stored overnight (4°C). Fixed cells were stained for DNA analysis by the propidium iodide method (Krishan, 1975). Briefly, cells were washed twice with cold TSE and then

resuspended by gentle vortexing into 1.0 ml of PI Solution (PI Solution is per 100 ml: 25 mg of propidium iodide (Sigma), 100 mg of sodium citrate, 10 mg of RNase A (Sigma), and 0.1% Triton X 100). Cells were incubated for at least 2 hrs in the dark and then passed through a 41-micron filter (Spectramesh; Spectrum). The cells were analyzed by flow cytometry on a Coulter EPICS -Profile II using Multicycle™ software (Phoenix Flow Systems), which allows for the removal of G1 doublets. 12.5×10^3 to 25×10^3 cells were sampled during each analysis. DNA content was determined by fluorescence and each analysis was normalized to the relative 2N DNA content of the G1 peak. Distribution of the S-phase was determined by a zero order function using Multicycle™ software (Phoenix Flow Systems). The scale of the cell number, ordinate axis, is determined by the relative distribution of fluorescent events, maximizing the cell cycle profile.

For bromodeoxyuridine (Budr) staining, cells were synchronized by serum starvation for 24 hours, then released by serum addition for 18 hours to enrich for cells in S phase. Cells were then labelled with 10 uM Bromodeoxyuridine (Sigma) and incubated at 39°C and 5% CO_2 for two hours. The media was replaced and the cells incubated at the indicated temperatures, then collected for cell cycle analysis using the manufacturers recommended protocol based on Beisker et al. (1987). Briefly, the cells were washed twice with PBS, trypsinized, centrifuged and washed twice with PBS/0.1% BSA. Final cell pellets were resuspended in 100 ul PBS/BSA, and fixed with 70% EtOH for 30 minutes on ice. Before staining for Budr with anti-Budr antibodies conjugated to Fluorescein isothiocyanate (FITC, Becton Dickinson), the cell pellet was acid denatured in 2N HCl/Triton X-100 for 30 minutes, then neutralized in 0.1 M $Na_2B_4O_7$, pH 8.5. 10^6 cells suspended in 0.5%

Tween20/1.0% BSA/PBS solution were used for each antibody reaction. Before analysis, cell pellets were resuspended in PBS containing 5 ug/ml propidium iodide as above. Cells were analyzed using linear scales on a Coulter EPICS Profile II. 7×10^3 to 8×10^3 cells were used in each analysis. Data was analyzed using the EPIC Cytologic DNA Package (Version 2.1). Background values of unlabelled cells were subtracted before each cell cycle analysis.

Immunoprecipitation and Densitometric Analysis.

p53 immunoprecipitations were performed as previously described (Hicks *et al.*, 1991). Briefly, [^{35}S]methionine labelled cells (1 h) were lysed for 30 min in 1.0 ml lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris-Hcl [Ph 8.0], 50μg aprotinin [Sigma]) on ice with intermittent shaking. Cellular debris was removed by centrifugation and the supernatant was precleared by a 40-min incubation in a resuspended pellet of a 10% suspension of crude *Staphylococcus aureus* (250 μl per sample, washed; Sigma). Following recentrifugation, 10^7 trichloroacetic acid precipitable counts of lysate supernatant was immunoprecipitated for 20 min with the appropriate monoclonal antibody: PAb240 a mutant p53 conformation specific monoclonal antibody (Gannon *et al.*, 1990), PAb246 a murine p53-specific monoclonal antibody sensitive to a conformational epitope on wtp53 protein but generally not reactive with mutant p53 protein species (Yewdell *et al.*, 1986), and either PAb419, a monoclonal antibody against SV40 Large T antigen (Harlow *et al.*, 1981) or 2 μg of immunoglobulin G2a murine polyclonal antibody (Sigma), as a control. Immune complexes were collected by a subsequent 40 min incubation with 100μl of a 3% suspension

of protein A Sepharose beads (preswollen and washed; Pharmacia). Samples were loaded onto a 12.5% polyacrylamide gel in sample buffer and in the presence of sodium dodecyl sulphate, and electrophoresed at 35mA. Fixed gels were treated with Enhance (Dupont), dried, and exposed to Eastman Kodak XAR-5 film with Lightening-Plus screens at -70°C. Densitometric scanning of autoradiographs was carried out on a BioRad model 620 Video Densitometer, and integrated using BioRad 1D Analyst™ software.

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FIGURE 1.

Temperature-dependent growth and survival of transfected REF52 cells.
4×10^4 log phase cells were seeded onto 60mm dishes and incubated at either 39°C (●) or 32.5°C (○), as indicated. Growth potential was determined by trypsinization and counting of the number of viable cells at the times shown in parallel cultures of each cell line. Each cell line and the transfected genes are shown. Standard error is the result of six independent experiments.

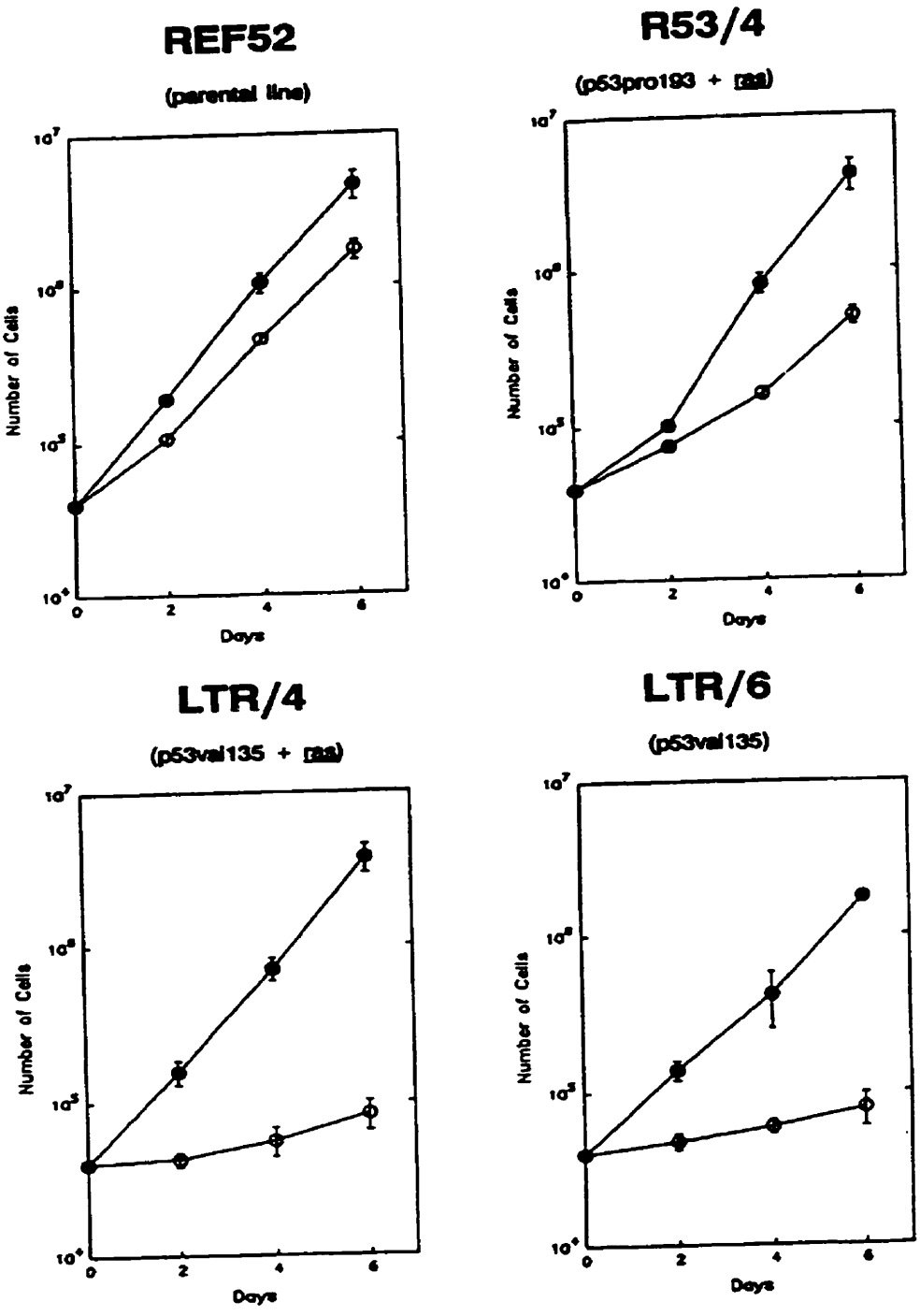
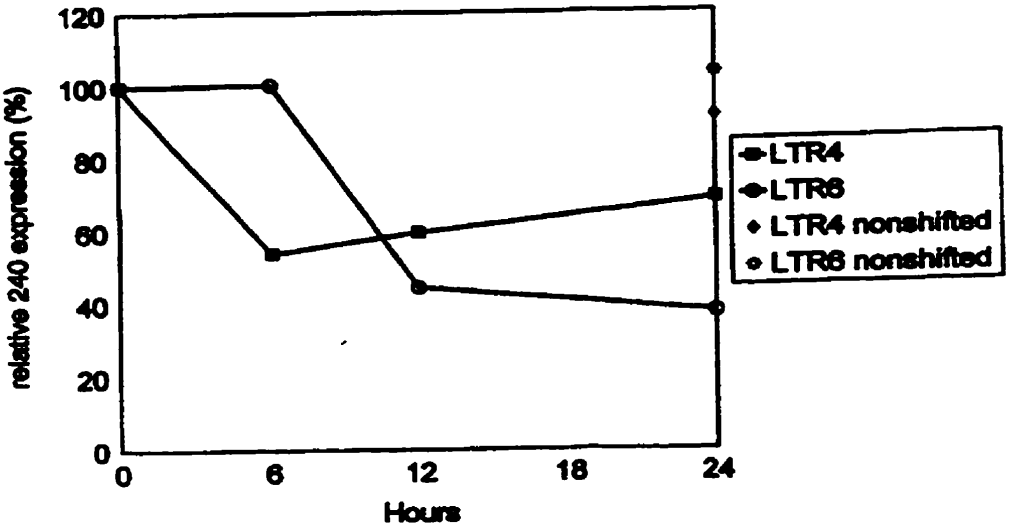

REF52
(parental line)
R53/4
(p53pro193 + ras)
LTR/4
(p53val135 + ras)
LTR/6
(p53val135)
Number of Cells
Days

FIGURE 2.

p53 epitope expression. 5×10^5 LTR/4(●), or LTR/6 (■) cells were incubated for 24 h at 39°C prior to being temperature-shifted to 32.5°C; time 0 h. Metabolically labelled cell lysates were immunoprecipitated, run on SDS-PAGE, and the resulting autoradiographs desitometrically analyzed. Shown here is the expression of PAb240 and PAb246 epitopes (panels A and B, respectively) which have been normalized to initial p53 expression (designated 100%), at time points up to 24 h post-temperature-shift. Additional symbols indicate the relative epitope expression of non-shifted cultures of LTR/4 (♦) and LTR/6 (✡) at 24 h.


120
100
80
60
40
20
0
relative 240 expression (%)
0
6
12
18
24
Hours
LTR4
LTR6
LTR4 nonshifted
LTR6 nonshifted

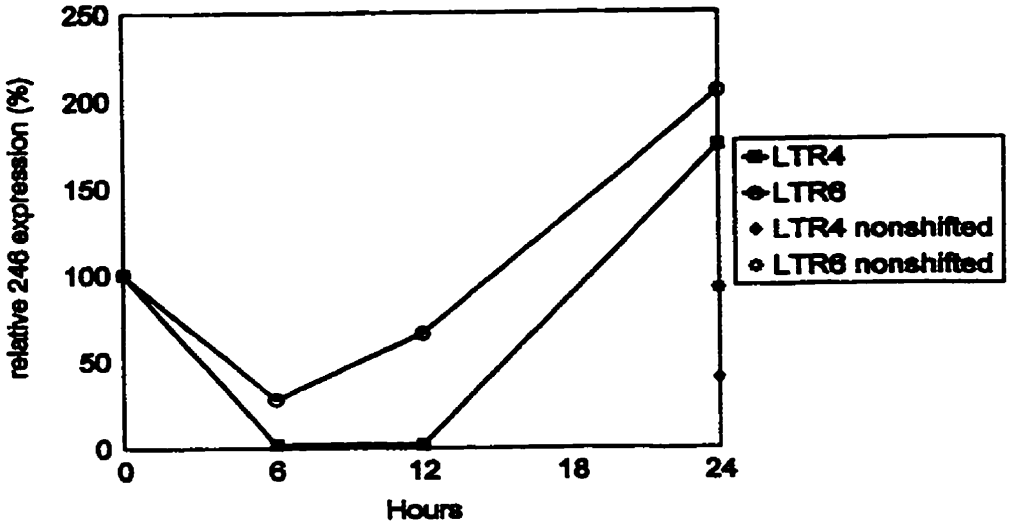
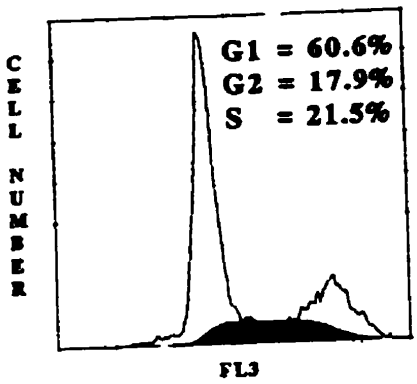
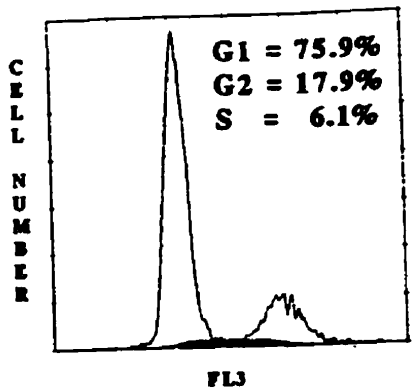

250
200
150
100
50
0
relative 246 expression (%)
0
6
12
18
24
Hours
LTR4
LTR6
LTR4 nonshifted
LTR6 nonshifted

FIGURE 3.

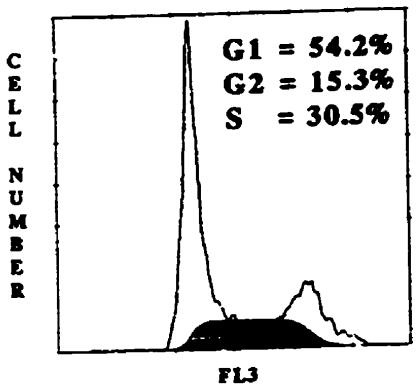
DNA analysis. Cell lines were incubated at the indicated temperatures and times, fixed, stained with propidium iodide, and analyzed on a Coulter EPICS II for DNA content. DNA fluorescence data were cell cycle analyzed using Multicycle™ software (Phoenix Flow Systems), which allows for the removal of G1 doublets during analysis. Distribution of S-phase (indicated by the shaded area) was determined as described in materials and methods. LTR4 - p53val^{135} + ras; LTR6 - p53val^{135}; REF52 - parental cell line.



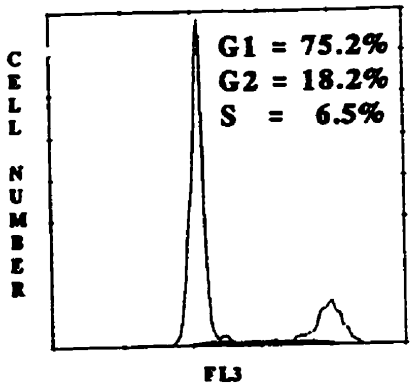
LTR/4 - 39°C



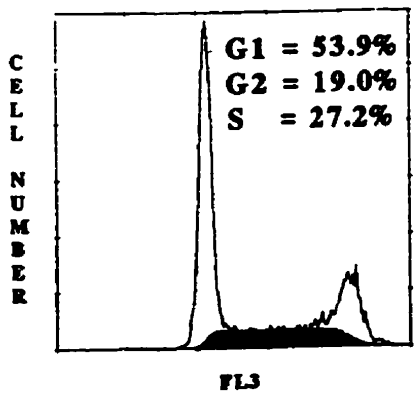
LTR/4 - 32.5°C



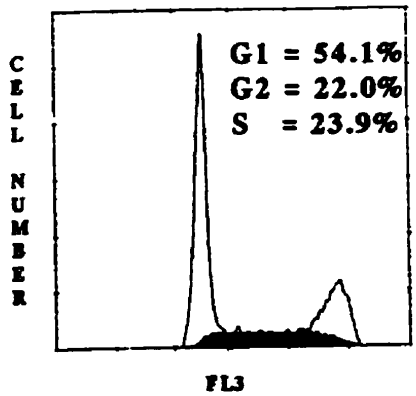
LTR/6 - 39°C



LTR/6 - 32.5°C



REF52 - 39°C

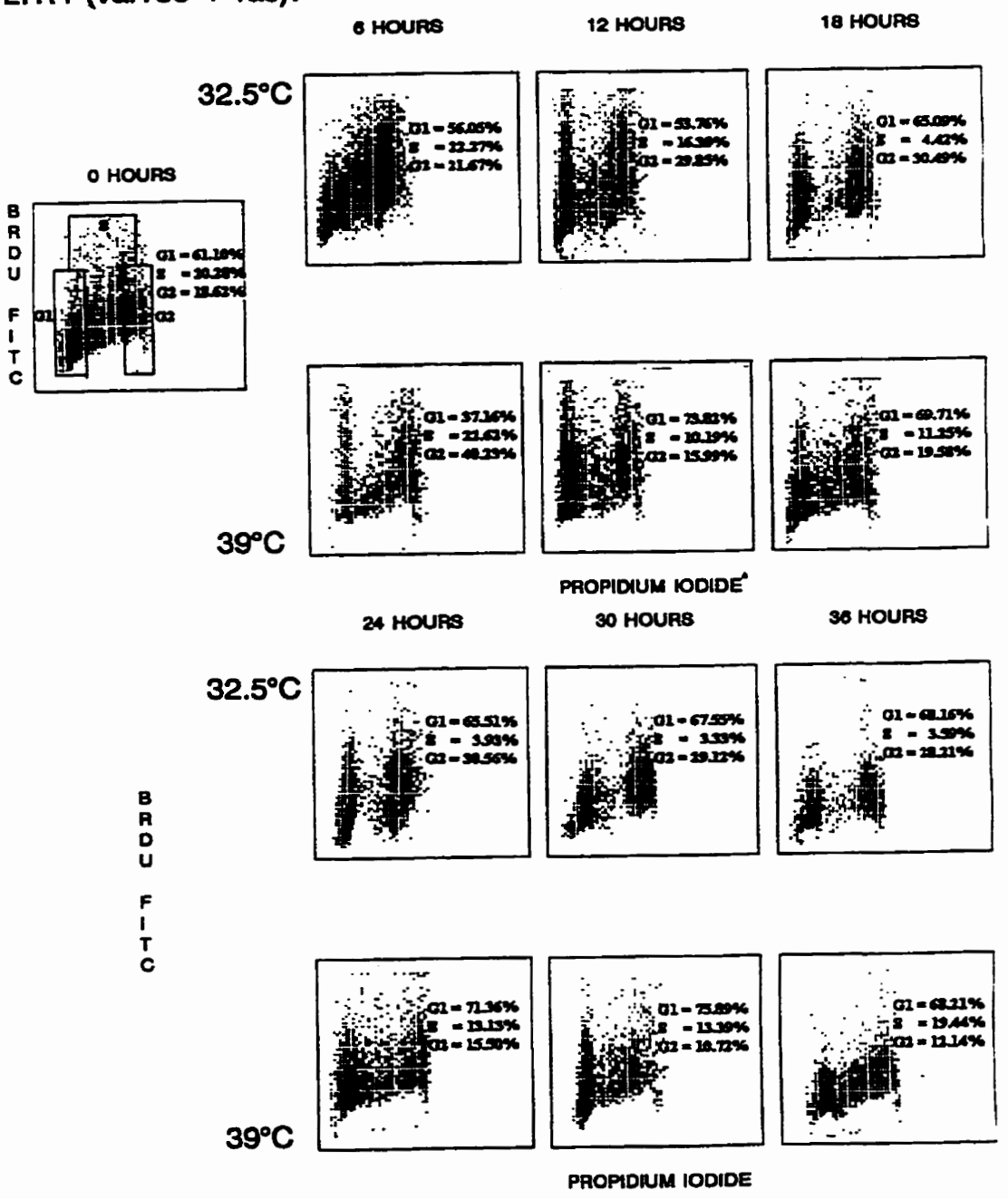


REF52 - 32.5°C

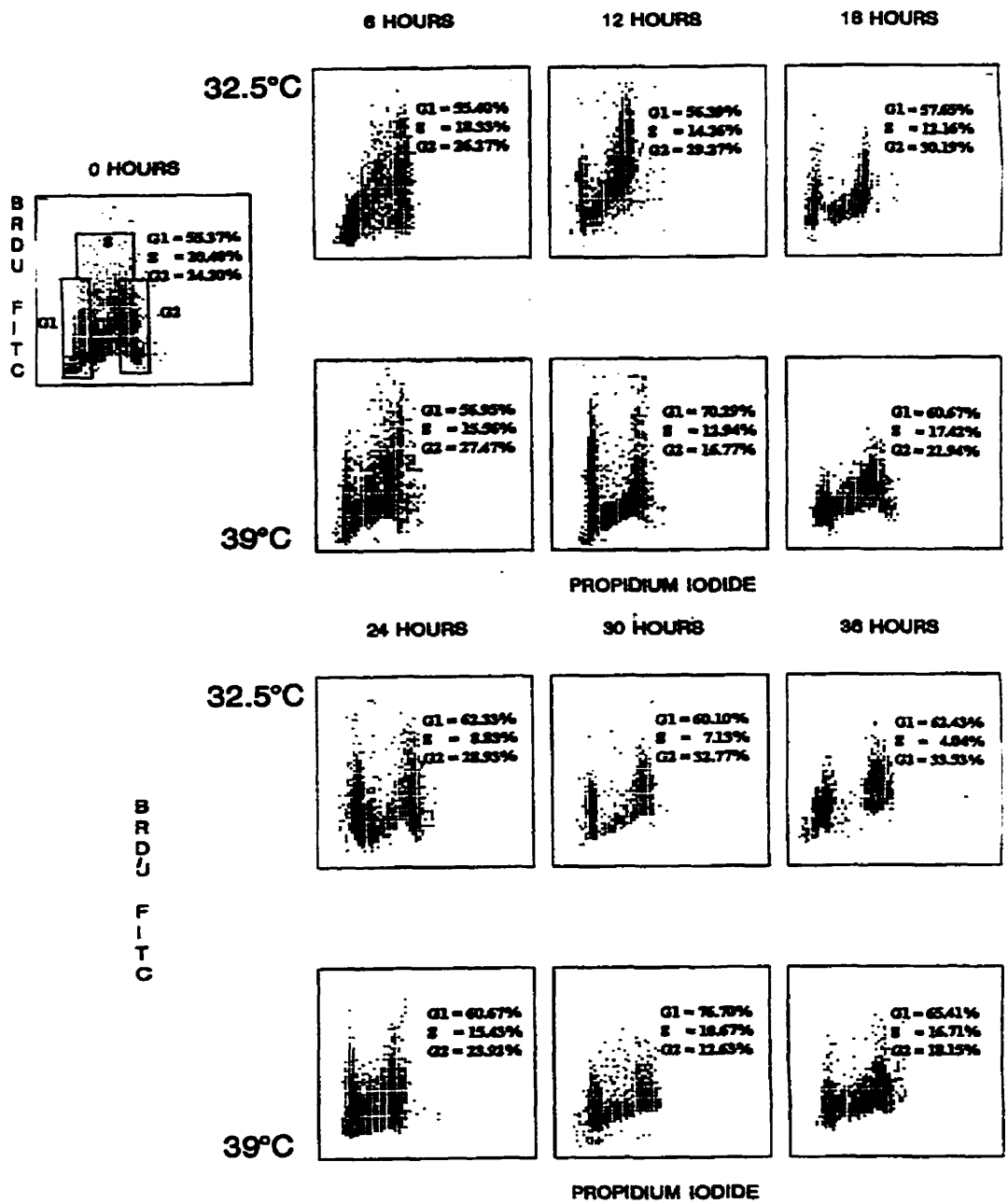
FIGURE 4.

Bromodeoxyuridine analysis. Synchronized cell lines (T0) were released from arrest for 18 hours, labelled for 2 hours with 10 μM Budr, then incubated at the indicated times and temperatures. Collected cells were denatured, neutralized and stained with anti-Budr FITC conjugated antibody as described in materials and methods. Data was analyzed using EPICS Cytologic DNA Package (Version 2.1), which includes the subtraction of unlabelled cells before cell cycle quantitation, and is expressed on a linear scale. Panel A: LTR/6; Panel B: LTR/4; Panel C: REF52.

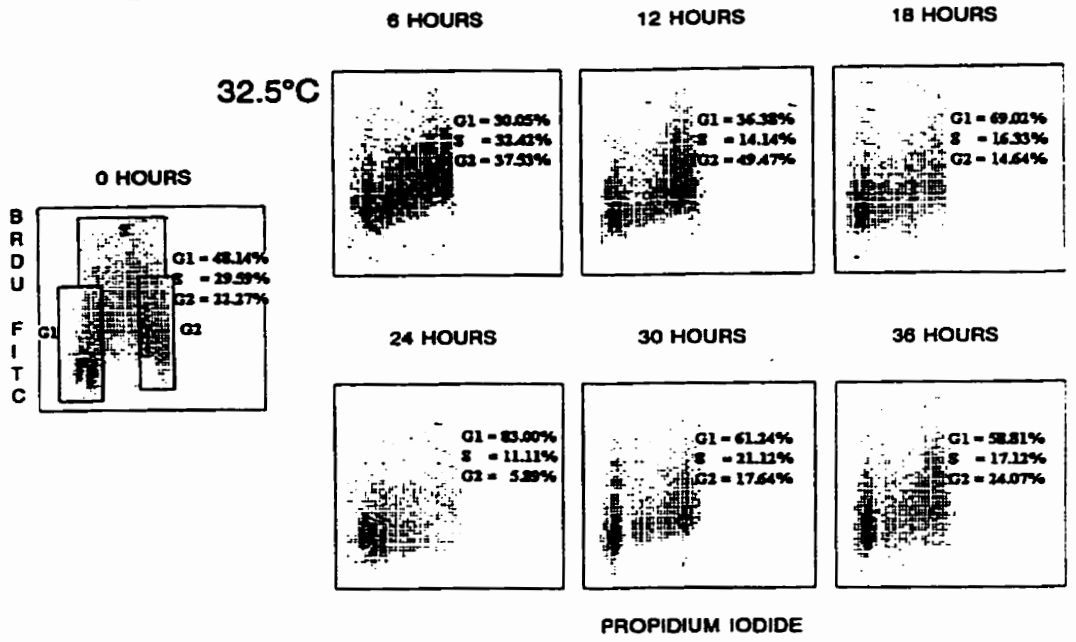
A LTR4 (val135 + ras):



B LTR6 (val135):



C REF52 (parental):



Mechanisms of Control over G2/M progression by p53

PREFACE

The following manuscript has been submitted to *Cell Growth and Differentiation*: Nancy Stewart, David W. Litchfield, Frixos Paraskevas, and Michael Mowat. 1998. Mechanisms of control over G2/M progession by p53.

This paper encompasses further characterization of the G2/M block induced by wild type p53 protein.

Mechanisms of control over G2/M by p53

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RUNNING TITLE: Cdc2 inactivation during p53 induced G2 arrest.

KEYWORDS: p53, G2 arrest, Cdc2, tyrosine 15, Cdc25C

ABSTRACT

Wild type p53 can induce cell cycle arrest at specific points in the cell cycle, in particular G1/S, an ability lost by most p53 mutants. We demonstrated previously that p53 can also induce cell cycle arrest at the G2 stage of the cell cycle (Stewart et al., Oncogene *10*, 109-115, 1995). Here we report further examination of this arrest point. Cdc2 kinase was found to be inactive in cells undergoing p53-induced G2 arrest. G2 arrest appeared to operate through a mechanism controlling the phosphorylation state of Cdc2. Cells arrested by p53 at G2 maintain phosphorylation of the regulatory tyrosine 15 amino acid on Cdc2 kinase. The levels of Cdc2 protein as well as the relevant cyclin, B1, were unaffected. Cyclin B1 protein in the G2 arrested population was found to surround the nucleus in the cytoplasm, consistent with the cells being paused in G2 phase. Caffeine, a known checkpoint inhibitor, added either both prior to or after establishment of G2 arrest could overcome the G2, but not the G1 arrest. This emphasizes the difference between the p53 induced G1 and G2 arrest points. One Cdc2 kinase regulatory phosphatase, Cdc25C, was hypophosphorylated in p53-induced G2 arrested cells. Release from G2 arrest by caffeine corresponded with the appearance of hyperphosphorylated Cdc25C. From this we conclude that p53 induces arrest G2 at least in part by controlling regulatory events allowing for phosphorylation and prevent activation of Cdc2 kinase, thus inhibiting completion of the cell cycle.

INTRODUCTION

In eukaryotes, cell proliferation is controlled by a family of cyclin-dependent kinases (cdks) (for a review, see: (King et al. 1994; Lew and Kornbluth, 1996; Fisher, 1997)). Cdc2 kinase was the first cyclin dependent kinase identified from work done on both *Saccharomyces cerevisiae* (Cdc28) and *Schizosaccharomyces pombe* (Forsburg and Nurse, 1991; Lew and Kornbluth, 1996). Cdc2 kinase is indispensable for passage through G2 and completion of mitosis (for review see (King et al. 1994; Grana and Reddy, 1995)). Together with it's cyclin partners B1 and B2 it also forms the maturation promoting factor (MPF) complex required for progression from G2 and through meiosis in *Xenopus* oocytes (Dunphy et al. 1988; Coleman and Dunphy, 1994). Cdc2 activation is controlled by a precise series of phosphorylation and dephosphorylation steps (at residues Thr 14, Tyr 15, and Thr 161), which occurs after binding to its cyclin partner B1 in S phase ((Brizuela et al. 1989; Gautier et al. 1988; Smythe and Newport, 1992), for a review, see (King et al. 1994)). Activation of Cdc2 requires phosphorylation at Thr 161, which is performed immediately after cyclin binding by the Cyclin-dependent kinase activating enzyme (CAK) (Solomon et al. 1993; Fesquet et al. 1993; Poon et al. 1993). Activity of the kinase complex is initially repressed by simultaneous phosphorylation of the Thr 14 and Tyr 15 residues by the Wee1 and Mik1 kinases, which phosphorylate the Tyr 15 residue after cyclin binding (Parker et al. 1992; Heald et al. 1993). Also, the recently discovered Myt1 kinase can phosphorylate Tyr 15 but preferentially acts on the Thr 14 residue (Liu et al. 1997). Dephosphorylation of Thr 14 and Tyr 15 by the Cdc25 phosphatases occurs late in G2, allowing for activation of Cdc2 and progression into mitosis (Gautier et al. 1991; Sebastian et al. 1993). Control over Cdc25C

activity in turn is negatively regulated by Chk1 kinase (O'Connell et al. 1997; Walworth et al. 1993; Al-Khodairy et al. 1994; Walworth and Bernards, 1996). Chk1 can also phosphorylate and activate Wee1 kinase (O'Connell et al. 1997).

Cells use a series of checkpoints to monitor completion of events during the cell cycle (such as completion of DNA synthesis) and delay progression into mitosis if circumstances require (for a review, see: (Nasmyth, 1996; Elledge, 1996; Sherr, 1996; Collins et al. 1997)). Wild type p53 is capable of inducing cell cycle arrest at specific points in the cell cycle after DNA damage, an ability lost by mutation of p53 (Kuerbitz et al. 1992; Elledge, 1996; Collins et al. 1997). The first p53 block characterized occurs at the R or restriction point before commitment to DNA replication (S phase) (Lin et al. 1992). P53 in part achieves this arrest through the transcriptional activation of a cdk inhibitor, $p21^{waf1/cip1}$ (el Deiry et al. 1993), which binds to and prevents activation of the cdk2-Cyclin E complex at late G1, and may also act to repress other cdk-cyclin complexs such as cdk4/6-Cyclin D (el Deiry et al. 1994; Harper et al. 1993; Xiong et al. 1993; Gu et al. 1993; Harper et al. 1995). p53 may also act through the transcriptional activation of the GADD45 gene (Kastan et al. 1992). GADD45 induces growth arrest in response to DNA damage when overexpressed (Zhan et al. 1994). Additionally, p53 may act in a transactivation-independent manner to induce growth arrest through a proline-rich motif near its amino-terminal end (Walker and Levine, 1996). Removal of this region does not affect p53's transactivational activity, but does reduce its ability to suppress colony formation in H1299 and SAOS-2 cell lines (Walker and Levine, 1996). Transcription-independent functions of p53 are necessary for the Gas1 gene, which produces a plasma membrane glycoprotein, to induce growth arrest (Del Sal et al.

1995). The proline rich motif of p53 has recently been shown to be required for induction of growth arrest by Gas1 (Ruaro et al. 1997).

We, amongst others, previously identified a second p53 induced arrest point found at G2 (Vikhanskaya et al. 1994; Stewart et al. 1995; Agarwal et al. 1995; Aloni Grinstein et al. 1995). P53 is also part of a negative growth control pathway, resulting in arrest at both G1 and G2/M, induced by overexpression of the *Ras* oncogene (Hirakawa and Ruley, 1988; Hicks et al. 1991). Negative growth regulation induced by overexpression of the *Ras* oncogene in the rat embryo fibroblast cell line REF52 can be rescued by the introduction of a dominant negative mutant p53 allele (Hicks et al. 1991). This Ras oncogene induced growth arrest is associated with cell senescence (Serrano et al. 1997). Inactivation of temperature sensitive SV40 Large T Antigen in conditionally immortalized fibroblasts induces senescence with arrest at both G1 and G2 parts of the cell cycle (Gonos et al. 1996).

Further, in p53 deficient cells disruption of the mitotic spindle complex with drugs that normally induce arrest at G2/M allows multiple rounds of DNA synthesis without cytokinesis (Cross et al. 1995). This suggests p53 also functions as part of mitotic spindle checkpoint control (ibid.). This p53 checkpoint function may operate independently of $p21^{waf1/cip1}$, as $p21^{waf1/cip1}$ deficient mice did not show any defect in spindle or G2 checkpoint control (Deng et al. 1995; Brugarolas et al. 1995). Possibly related to this arrest is p53's control over centrosome duplication. Fibroblasts from p53 deficient mice show multiple copies of centrosomes that result in abnormal segregation of chromosomes (Fukasawa et al. 1996). P53's control over this spindle fibre checkpoint may be important in preventing endoreduplication, and the generation of aneuploid (including polyploid) cells often observed

in p53 deficient cell populations (Harvey et al. 1993; Lee and Kirschner, 1996; Galipeau et al. 1996).

Cells deficient in p53 still show a G2/M arrest after irradiation (Kuerbitz et al. 1992). However, there is evidence to suggest that other mechanisms function to regulate Cdc2 kinase and induce G2/M arrest, which may or may not require p53 for their action. Activation of the Lyn tyrosine kinase induces a G2/M arrest in response to irradiation in haematopoietic cells (Kharbanda et al. 1994; Kharbanda et al. 1996; Uckun et al. 1996). Ionizing radiation (IR) induces rapid activation of Lyn kinase, which in turn can bind to Cdc2 kinase and result in inhibitory phosphorylation at the Tyrosine 15 residue of Cdc2 kinase (Kharbanda et al. 1994; Kharbanda et al. 1996; Uckun et al. 1996). Further, IR fails to cause tyrosine phosphorylation of Cdc2 kinase or G2 arrest in $Lyn^{-/-}$ B-cell precursors (Uckun et al. 1996). To date no evidence has been provided to suggest p53 works on this pathway. Similarily, c-Abl tyrosine kinase is also activated in response to DNA damaging agents such as irradiation (Kharbanda et al. 1995), and the BCR-ABL protein chimera can induce a G2/M growth arrest when expressed at elevated levels in haematopoietic cells (Kharbanda et al., 1996). This protects cells from radiation and chemotherapeutic induced apoptosis (ibid). Overexpression of c-Abl also induces growth arrest at G1 through binding to p53 protein (Sawyers et al. 1994; Goga et al. 1995). Binding of active c-Abl to p53 is also required for the induction of growth arrest in response to genotoxic drugs or irradiation and is associated with down regulation of cdk2 kinase activity, but does not require transactivation of the $p21^{waf1/cip1}$ gene (Yuan et al. 1996; Yuan et al. 1996).

Recent work has suggested that control over Cdc25C may also have a central role in

the response to DNA damage at G2. After DNA damage in yeast, inhibitory phosphorylation of Cdc2 on tyrosine 15 occurs, involving activation of Wee1/Mik1, inactivation of Cdc25C and arrest in G2/M (Rhind et al. 1997). This G2/M arrest point in yeast is mediated by the Chk1 kinase, which is essential for this arrest (O'Connell et al. 1997; Walworth et al. 1993; Al-Khodairy et al. 1994; Walworth and Bernards, 1996). Human Cdc25C becomes phosphorylated on Ser216 after DNA damage, which is an inactivating phosphorylation (Rhind et al. 1997; Sanchez et al. 1997; Peng et al. 1997). Both yeast and human Cdc25C are phosphorylated by the Chk1 kinase *in vitro* and *in vivo* (Furnari et al. 1997; Sanchez et al. 1997; Peng et al. 1997). This phosphorylation event is required for DNA damage induced arrest (ibid). In addition, human cells expressing a non-phosphorylatable Ala216 mutant are defective for G2/M DNA arrest after DNA damage (Peng et al. 1997). Together these results suggest phosphorylation at Ser-216 on Cdc25 by Chk1 kinase is required for DNA damage induced growth arrest at G2 . Interestingly, Chk1 can also phosphorylate and activate Wee1 kinase, providing a second possible mechanism for Cdc2 inactivation (O'Connell et al. 1997). Phosphorylation of Ser-216 permits binding of members of the 14-3-3 protein family (Peng et al. 1997). This also inhibits Cdc25 activity, possibly by sequestering Cdc25C and preventing it from activating Cdc2 (ibid). How this regulatory event relates to the Lyn and/or c-Abl induced G2 arrest pathways remains unknown at present.

The presence of at least two complementary and/or independent pathways used by cells at G2 in response to genotoxic stress will make the effects of p53 on G2 arrest difficult to discern. Very little is known about the possible mechanisms of p53 induced G2 cell cycle

arrest. With that in mind, we examined the mechanisms for the p53 induced G2 arrest. To accomplish this we used the REF52 cells transfected with the temperature sensitive p53val^{135} mutant (Stewart et al. 1995). Other people have shown that in different cell systems (M1 myeloblastic leukemia cells), p53 is not involved in irradiation induced growth arrest but is involved in apoptosis (Guillouf et al. 1995). Our system allows us to study the response of cells during G2 caused by overexpression of p53, without the potential induction of other G2 arrest pathways or apoptosis when using DNA damaging agents such as irradiation. We chose to look at the expression and regulation of Cdc2 kinase. This is important as the DNA replication checkpoint has not always been found to be dependent on tyrosine phosphorylation of Cdc2 kinase. In *Xenopus*, there has been a report of replication checkpoint-dependent suppression of Cdc2-Cyclin B by a unidentified titratable inhibitor which is regulated by the presence of unreplicated DNA (Kumagai and Dunphy, 1995). This inhibitor can be inhibited by catalytically inactive Cdc2-Cyclin B (ibid). A membrane associated Cdc2 inhibitor complex has been recently identified from interphase Xenopus extracts, but it is unknown if it plays any role in this checkpoint (Lee and Kirschner, 1996).

We report that p53 induced arrest at G2 involves maintenance of the Cdc2 kinase complex in an inactive form. This inactivation is most likely due to phosphorylation of Cdc2 at tyrosine 15 and not due to a lack of Cdc2 kinase protein. Addition of caffeine to G2 arrested cells releases them from this arrest and allows them to cycle until the p53 induced G1 arrest point. Cdc25C protein is hypophosphorylated during G2 arrest, and becomes phosphorylated upon the addition of caffeine, suggesting inactivation of Cdc25C may be one way p53 influences phosphorylation of tyrosine 15 on Cdc2.

RESULTS

G2 Synchronization of REF52 cells

We created several cell lines from the REF52 parental line using a plasmid expressing p53val^{135} (pGp53VS; (Meek and Eckhart, 1990), a temperature sensitive mutant with wild type conformation at 32°C, and mutant at 37.5°C). This construct expresses both the major and the splice variant forms of p53. In order to enrich for cells at the p53 induced G2 block we took two stratagies. First, a mixed cell population containing cells at both G1 and G2 arrest points were separated by centrifugal elutriation. This allowed isolation of cells at the p53 induced G2 arrest point up to 80% of the total sample (Figure 1a). We occasionally notice a greater than 4N population in the N223E1 cells (Figure 1a). This may result from overexpression of mutant p53, as loss of normal p53 function is associated with aneuploidy, possibily due to abnormal centrosome duplication and chromosomal segregation (Fukasawa et al. 1996; Fukasawa et al. 1997).

This method, however, was unsuitable for examination of events occurring in the cells as they approached and entered p53 induced G2 arrest. For these experiments we used a double synchronization approach. Cells were first synchronized by serum starvation for 24 hours, then released with serum and arrested in early S phase with hydroxyurea for a further 24 hours. From prior experience we knew that the arrest point for hydroxyurea occurred after the p53 induced G1 block (unpublished results). Before being released from the drug arrest the cells were shifted to 32°C for a minimum of 8 hours to allow turn over of the p53 protein species from mutant conformation to wild type. The cells were then released from hydroxyurea arrest and collected periodically for analysis up to 2 days into the

G2 arrest point (Figure 1b). One such population (79% arrested in G2 for 48 hours, as determined by flow cytometry) was used to examine the morphology of cells arrested by p53 at G2. Examination of the arrested cells failed to demonstrate any significant number of obvious mitotic figures, the majority appearing to contain interphasic nuclei (Figure 1c).

Cdc2 kinase is inactive during G2 arrest

Cdc2 is the primary kinase responsible for transition through late S/G2 and into mitosis. Accordinly, we examined Cdc2 kinase activity during G2 arrest. Cdc2 kinase was immunoprecipitated from cell extracts obtained from p53 arrested and control cell populations and assayed for kinase activity. As demonstrated in Figure 2, no appreciable Cdc2 kinase activity was detected in cells arrested either at G1 or for 1 or 2 days at G2 by p53, as compared to control cells arrested with nocodozole which show elevated Cdc2 kinase activity.

Cdc2 protein expression is not affected during G2 arrest

We next wanted to verify that the lack of active Cdc2 in our G2/M arrested cells was not due to loss of Cdc2 expression. Protein lysates from both cycling cells and p53 arrested cells were obtained and tested by western immunoblotting to determine changes in the expression of Cdc2 protein. As shown in Figure 3, the level of protein expression of Cdc2 remains relatively consistent throughout the cell cycle and during both p53 induced G1 and G2/M arrest points. This is consistent with other studies that demonstrated that expression of Cdc2 kinase protein normally remains constant regardless of position in the cell cycle

(Welch and Wang, 1992). Thus, we conclude Cdc2 kinase inactivation is not occuring through loss of Cdc2 protein expression.

Expression of Cyclin partners in G2 arrested cells

Another possible mechanism for inactivating Cdc2 kinase would be to affect expression of its cyclin partner, Cyclin B1. Cyclin B1 expression was examined by western immunoblotting in cycling and p53 arrested cells. As demonstrated in Figure 4a, Cyclin B1 is expressed at comparable levels both during the initial stages of G2 arrest in p53 blocked REF52 cells, as well as a mixed population of non-arrested cells (top panel). This expression dropped slightly at 12 hours into the G2 arrest but remained steady for up to 2 days. In contrast, Cyclin B1 levels in the cycling parental REF52 cells fell off after 8-12 hours when most cells were either in or past mitosis, and did not begin to recover until their reentry into S phase (24 hours, Figure 4a bottom panel). Overall, the existence of cyclin B1 in the p53-induced G2 arrested cells suggests that the absence of Cdc2 activity does not reflect an absence of its cyclin partner.

The protein levels of two other cyclins, A and E were also examined. Cyclin A expression peaked within 4 hours of release in the G2 arresting cells, then fell off and failed to recover later into G2 arrest (Figure 4b, top panel). In contrast, Cyclin E protein expression becomes completely deregulated, with levels increased in both arrested and cycling N223E1 cells regardless of their position in the cell cycle (Figure 4b, bottom panel). Uncoupling of Cyclin E expression to cell cycle position has been previously reported in some tumor cell lines (Darzynkiewicz et al. 1996).

We also wished to confirm that Cyclin B1 was bound with Cdc2 kinase during G2 arrest. Cell cultures were synchronized and collected as above. Cdc2 kinase was immunoprecipitated with Cdc2 antibodies conjugated to Sepharose-A beads, separated and examined on a western blot for both Cyclin B1 and Cdc2 protein. As shown in Figure 4c, a band corresponding to Cyclin B1 can clearly be seen in the arrested cell populations. This band is lost if the Cyclin B1 antibody is first blocked with its competitive peptide (Figure 4c, bottom panel). The level of Cyclin B1 does diminish somewhat over time (24-48 hours), which may indicate some disassociation of the complex later into p53 induced G2 arrest. Nonetheless, the present of a coimmunoprecipitating Cyclin B1 band indicates that Cdc2 and Cyclin B1 are associated during G2 arrest.

Cdc2 protein phosphorylation is altered in arrested cells

The Cdc2/Cyclin B1 complex requires dephosphorylation of Cdc2's tyrosine residue 15 for activation and progression of the cells through G2 and mitosis (Gautier et al. 1991). To examine Cdc2 phosphorylation as cells progressed into G2 arrest, cultures were synchronized at S phase with hydroxyurea then released at 32°C as previously described, and collected at the specified times. Cdc2 kinase was then immunoprecipitated, separated and western blotted, and phosphorylation at Tyr 15 examined using a phospho-specific Cdc2 (Tyr 15) antibody (New England Biolabs, Figure 5). In parental REF52 cells passing through G2/M, Cdc2 protein phosphorylation peaks at 8 hours past the hydroxyurea release (Figure 5b), corresponding to when most cells were in late S/G2 (approximately 73%), and decreased rapidly thereafter as the cells reentered G1. In contrast, Cdc2 protein in the G2

arrested cells did not become dephosphorylated upon release from hydroxyurea and entry into the G2 arrest. In these cells, Cdc2 remained phosphorylated at Tyr15 for up to 2 days, the level dropping slightly at later times (Figure 5a). These results indicate that Cdc2 kinase is inactivated in p53 arrested cells due to phosphorylation at Tyr 15.

Caffeine overcomes arrest in G2 by p53

Caffeine is a known inhibitor of G2 delay and arrest points, as well as an inducer of premature mitosis (Schlegel and Pardee, 1986). It has been previously reported that caffeine treatment can activate Cdc2 kinase (Schlegel and Pardee, 1986; Lock et al. 1994; Powell et al. 1995; Yao et al. 1996). We wished to test whether caffeine could overcome the G2 block induced by p53. Cells were synchronized at S phase by hydroxyurea as previously indicated and released into G2 arrest conditions, with and without caffeine. As demonstrated in Figure 6a, most cells in the presence of 2 mM caffeine failed to stop at G2 and continued through mitosis until they reached G0/G1. The cells did not continue to cycle, the majority remaining in G1 for up to 3 days (Figure 6b, Growth curves). Addition of caffeine to cycling populations left at 37.5°C had no effect (data not shown).

We also tested whether caffeine could overcome an established p53 G2 arrest. Cells were arrested at G2 for 1 day, then 2 mM caffeine was added and cell cycle position determined (Figure 7a). Similar to previous experiments, these cells also exited the G2 arrest and progressed until G1 before arresting again. Addition of caffeine to cells arrested by p53 at the G1 point failed to overcome the G1 arrest (Figure 7b).

Expression of Cdc25C

Removal of the phosphate on Tyr 15 of Cdc2 kinase requires the action of the Cdc25C phosphatase prior to mitosis (Gautier et al. 1991; Sebastian et al. 1993). We wanted to determine the status of Cdc25C in the p53 G2 arrested cells. The other two isoforms of Cdc25, A and B were not examined as they are active at different points in the cell cycle. Cdc25A appears to function at G1/S progression (Jinno et al. 1994; Hoffman et al. 1994) and Cdc25B functions during prophase, inducing microtubule nucleation before mitosis by activating cytoplasmic Cdc2/Cyclin B (Gabrielli et al. 1996).

Cdc25C is activated by phosphorylation which is detected as a substantial shift (54-57 kd to 66 kDa) on protein gels (O'Connor et al. 1994; Garcia and Cales, 1996). To determine if Cdc25C phosphatase is active in p53 arrested cells we looked for this shift in Cdc25C protein expression in our p53 induced G2 arrested populations. Cell lysates from G2 arrested populations were obtained as indicated in Figure 5, and cycling cells were compared. As demonstrated in Figure 8, we were unable to detect a shift from lower to higher molecular weight species in the G2 arrested populations (top panel), while a shift was present in cells that were near or entering mitosis after release from hydroxyurea (middle panel). There was also loss of the middle band(s) associated with the inhibitory Chk1 phosphorylation event after 8 hours in the cycling population. The Cdc25C status was also examined in cells prevented from arresting at G2 by the addition of caffeine. Here we saw the appearance of the higher molecular weight species of Cdc25C within 2 hours of the addition of caffeine, suggesting Cdc25C is activated in these cells. We also noticed the gradual but not complete loss of the middle band, mimicking that seen in the cycling cells.

Therefore, we conclude that during G2 arrest Cdc25C is hypophosphorylated, suggesting it may be inactive.

Cyclin B1 is located outside the nucleus in G2 arrested cells

In an attempt to better understand where in the cell cycle our G2 population is arrested, we performed immunofluorescence to determine the cellular location of Cyclin B1. Cyclin B1 accumulates in the cytoplasm during S phase, surrounding the nucleus by G2. The protein enters into the nucleus upon mitosis before nuclear lamina breakdown (Pines and Hunter, 1991; Li et al. 1997; Widrow et al. 1997). It remains in the nucleus until the metaphase-anaphase transition where it is rapidly degraded and lost prior to telophase (ibid; Kakino et al. 1996). As demonstrated in Figure 9 (Panel E), cells arrested in G2 showed Cyclin B1 cytoplasmic staining concentrated around the nucleus, resembling that seen during G2 (Pines and Hunter, 1991; Li et al. 1997; Widrow et al. 1997). In contrast, cells arrested by p53 at G1 showed a diffuse staining throughout the cytoplasm (Panel D). Control cells arrested at mitosis by nocodozole exhibited an intense nuclear staining (Panel A), whereas cells arrested at G1/S by hydroxyurea (Panel B) showed a diffuse cytoplasmic staining. These results suggest that the G2 arrested cells have stopped at a point in G2 before mitosis, and p53 may directly or indirectly prevent transport of Cyclin B1 to the nucleus.

DISCUSSION

In this report we show that Cdc2 kinase is inactivated during arrest at G2 induced by expression of wild type p53, and this inactivation is most likely due to maintenance of phosphorylation at Cdc2's tyrosine 15 residue. The lack of Cdc2 activity is not due to an absence of protein expression. This is in contrast to a recent report that suggests p53 can downregulate Cdc2 protein/mRNA levels through $p21^{waf1/cip1}$ in response to ionizing radiation in cells synchronized at G0-G1 by density inhibition (Azzam et al. 1997). Initial expression of its cyclin partner B1 was comparable to Cyclin B1 levels in control G2 cells, although there was a modest drop in Cyclin B1 protein expression later in G2 arrest. The nature of our experiments did not allow us to address whether phosphorylation of Thr 14 is also important for arrest by p53 at G2, thus this also remains a possibility. Addition of caffeine to p53 induced G2 arrested cells released them from the arrest and allowed them to pass through mitosis and into G1 where they arrested. Cdc25C was hypophosphorylated and singly phosphorylated by Chk1 kinase in G2 arrested cells and became phosphorylated upon the addition of caffeine, suggesting Cdc25C inactivation is important for maintaining tyrosine 15 phosphorylation on Cdc2.

We assume that the cells are being arrested at G1 by p53 induction of $p21^{waf1/cip1}$ since these experiments were conducted at the restrictive temperature. Induction of $p21^{waf1/cip1}$ expression is one of the mechanisms used by p53 for cell cycle control (el Deiry et al. 1993), and may be important for p53 dependent reduction of Cdc2 kinase protein/mRNA levels after ionizing radiation at G0/G1 (Azzam et al. 1997). Activation of $p21^{waf1/cip1}$, however, is probably not part of the mechanism employed by p53 in inducing arrest at G2. $P21^{waf1/cip1}$

has been reported to be a poor inhibitor of Cdc2/Cyclin B kinase activity (Poon et al. 1996). Further, cells from $p21^{-/-}$ mice still possess functional G2 and spindle checkpoint controls (Brugarolas et al. 1995; Deng et al. 1995), suggesting that any effects p53 has on this point of the cell cycle can occur without the presence of $p21^{waf1/cip1}$. During our experiments where we had immunoprecipitated [^{35}S] labelled Cdc2 from our G2 arrested cell populations, we failed to observe any bands at 21 kD which might indicate the presence of $p21^{waf1/cip1}$ (unpublished data). Addition of caffeine that overcomes p53 G2 arrest presumably would not affect the binding of $p21^{waf1/cip1}$ since the p53 G1 arrest remains intact. These results support the finding that $p21^{waf1/cip1}$ is not a significant part of the inhibition of Cdc2/Cyclin B1 complex during p53 induced G2 arrest.

Inactivation of Cdc2 kinase by phosphorylation at its Tyr 15 residue is a common feature of several G2/M DNA damage checkpoints (Ye et al. 1997; Wang et al. 1996; Lock et al. 1994; Herzinger et al. 1995), making it reasonable to suppose that p53 might employ a similar pathway. Arrest at the G2/M arrest point by DNA damage can occur without the presence of p53 (Kuerbitz et al. 1992). However, it is interesting to note that in at least one case the presence of p53 protein can make cells more resistant to abrogation of the G2 DNA damage checkpoint (Wang et al. 1996). Compared to cells lacking functional p53, human lymphoma CA46 cells carrying functional p53 protein induced to arrest at G2 by DNA damaging agents such as cisplatin were more resistant to release and subsequent cell killing by UCN-01, a protein kinase inhibitor (Wang et al. 1996). Further, immortal Li-Fraumeni fibroblasts lacking wild-type p53 display a defective G2 checkpoint response to ionizing radiation (Paules et al. 1995). This suggests that p53, although not necessary for all

responses by the cell to DNA damage at G2 may play a separate or complementary role. Indeed, a recent report looking at isogenic human fibrosarcoma cell lines suggests that p53 may monitor DNA damage throughout the cell cycle and determine whether the cells need to arrest at the upcoming cell cycle block (G1 or G2) to repair their DNA or continue on (Pellegata et al. 1996).

Inactivation of Cdc2 kinase by phosphorylation at Tyr 15 could be achieved by either activation of the Wee1/Mik1 kinases or inactivation of the Cdc25B/C phosphatases. Inactivated Cdc25C phosphatase has been previously implicated in G2 arrest induced by nitrogen mustard (O'Connor et al. 1994). Inactivation of Cdc25C is also important during G2 arrest and endoreduplication in megakaryoblastic cells (Garcia and Cales, 1996). We determined that induction of the p53 induced G2 arrest can be overcome by the addition of caffeine, a known inhibitor of other G2 phase arrest mechanisms (Powell et al. 1995; Lock et al. 1994). In those cases, caffeine results in the dephosphorylation of Cdc2 kinase and subsequent activation. Although the precise mechanism for caffeine's action is unknown at present, indirectly it allows for activation of Cdc25 phosphatases or inactivation of Wee1 kinase or other kinases through phosphorylation. The results from our Cdc25C protein analysis suggests that Cdc25C is hypophosphorylated in p53 G2 blocked cells. Addition of caffeine resulted in the appearance of the upper migrating band associated with phosphorylated (via Plk1) activated form of Cdc25C. We also noticed that the second Cdc25C band, associated with the Chk1 inhibitory phosphorylation event (Walworth et al. 1993; Al-Khodairy et al. 1994; Walworth and Bernards, 1996), was present in the G2 arrested populations. This band also gradually disappeared after the addition of caffeine

(Figure 8). As indicated, Chk1 is associated with G2 damage response (O'Connell et al. 1997; Walworth et al. 1993; Al-Khodairy et al. 1994; Walworth and Bernards, 1996).

During the preparation of this manuscript a potential molecular mechanism of G2/M control by p53 was described involving 14-3-3σ protein. The 14-3-3σ gene is upregulated by irradiation in human colorectal cell lines (Hermeking et al. 1997). This response is mediated by wild type p53, and results in arrest at G2 prior to mitosis (ibid). 14-3-3σ was not induced in cells lacking wild type p53 (ibid). 14-3-3 has been shown to bind to Cdc25C after the inhibitory phosphorylation on the Ser216 residue (Peng et al. 1997). This event does not inhibit Cdc25 activity, but may sequester Cdc25C in the cytoplasm, preventing its activation of Cdc2 in the nucleus (ibid). These results point to a potential mechanism of control by p53 over Cdc25C and thus Cdc2 activity, but along with our results pose a number of questions. 14-3-3 binding of Cdc25C requires Chk1 phosphorylation of Ser216 (Peng et al. 1997). As mentioned, our results indicate this event has occurred in the p53 induced G2 arrested cells. Does p53 also induce Chk1 action? What role do the Polo kinases (Plk1) have in G2 arrest? Our results suggest that in cells arrested by p53 at G2, Cdc25C has not undergone the activating phosphorylation event associated with Plk1. Our results also indicate that Cyclin B1, and presumably Cdc2 as the two proteins appear to be bound (Figure 4C). Cyclin B1 also remains localized in the cytoplasm around the nucleus (Figure 9). Whether Cdc2 phosphorylation also influences Cdc2/Cyclin B1 localization and thus its activity is not known at this time. It is also necessary to address what effect 14-3-3σ upregulation might have on Wee1 function, as Wee1 is phosphorylated by Chk1 and is bound by 14-3-3, which may affect both its activity and localization in the cell (Honda et al.

1997; O'Connell et al. 1997).

The results from our experiments suggest that phosphorylation is an event important for the arrest by p53 at G2. Expression of a Cdc2 phosphorylation site mutant Cdc2AF in Hela cells during early S-phase did not induce premature mitosis, but did induce mitosis in 25% of cells that had progressed through S phase (Jin et al. 1996). This suggests regulation of Cdc2 kinase activity via phosphorylation may be more important later, rather than during S phase checkpoints. This may also explain the difference seen in Cdc2 protein expression between our cells arrested by p53 at G2 and the results of Azzam et al. (Azzam et al. 1997). They reported that Cdc2 protein/mRNA expression was downregulated in a p53 dependent manner after ionizing radiation in both human and rodent fibroblasts synchronized at G0/G1 (ibid). This suggests that the regulation of steady state levels of Cdc2 protein may be more important at G0/G1 to prevent Cdc2 activity than phosphorylation.

Cdk2 kinase activation has also been suggested as an indirect positive regulator of the Cdc2-Cyclin B complex from work using *Xenopus* cell free extracts (Guadagno and Newport, 1996). Cdk2 may act in a positive fashion on Cdc25C, as Cdk2-Cyclin E phosphorylates and activates Cdc25C *in vitro* (Izumi and Maller, 1995). One possibility could be that p53 exerts its control by repressing Cdk2 activity through $p21^{waf1/cip1}$, which in turn might down regulate Cdc2 kinase activity. However, a recent article reported that the injection of $p21^{waf1/cip1}$ into intact oocytes failed to prevent the oocytes from completing meiosis properly, arguing against Cdk2 kinase activation of the Cdc2-Cyclin B complex factor (Furuno et al. 1997). Also, $p21^{waf1/cip1}$ deficient mice still possess a G2 and spindle checkpoint control (Brugarolas et al. 1995; Deng et al. 1995). Further, overexpression of

Cdk2 on its own has already been demonstrated not to overcome p53 mediated growth arrest (Latham et al. 1996), making it unlikely Cdk2 has any direct role in p53 control over Cdc2 kinase.

We still have an imprecise understanding of where in G2 the cells are blocked. One group has suggested that p53 dependent cell cycle arrest after mitotic spindle damage results in arrest at a G1-like state without prior mitosis (Minn et al. 1996). FL5.12 cells expressing Bcl-x_L undergo p53 dependent cell cycle arrest after mitotic spindle damage. This arrest did not occur in mitosis, instead the cells proceeded into G1 without mitosis where they were arrested by p53 (ibid). For our cells, Cyclin B1 expression indicates they have probably passed into the later portion of G2, but the failure to observe any obvious mitotic figures, plus the continued expression of Cyclin B1, suggests they have not entered into mitosis. The results from our immunofluorescent study of Cyclin B1 location (Figure 9) suggests that the cells have arrested in G2 before mitosis, as the protein remains in a concentrated "halo" around the nucleus. The cells do express high levels of Cyclin E. However, basing the cell cycle status on the expression levels of cyclins such as Cyclin E can be deceptive, as we as well as others have noticed that Cyclin E levels often become deregulated in transformed cells relative to the cell cycle position (Darzynkiewicz et al. 1996), making Cyclin E unreliable as a position marker. The presence of wild type p53 in fact can prevent DNA rereplication in fibroblasts which have been arrested with nocodozole and colcemid, drugs which interfere with spindle assembly (Di Leonardo et al. 1994). Finally, when caffeine is added to G2 arrested cells, they pass through mitosis within a relatively short period of time and cycle until they stop at the p53 induced G1 arrest point (Figures 6 and 7), suggesting that

they had not originally stopped at a G1 like state, and that the p53 induced G1 and G2 arrest points work through different mechanisms.

In conclusion, we find that induction of arrest at G2 by p53 involves the inactivation of Cdc2 kinase by phosphorylation at its Tyr 15 residue. This arrest point can be overcome by the addition of caffeine, allowing the cells to pass through mitosis and into G1 where they undergo p53 induced G1 arrest.

MATERIALS AND METHODS

Cells and Culture

REF52 cells are an established rat embryo fibroblast line (Franza et al. 1986) and were a gift from H. Earl Ruley. The following lines were derived in our laboratory and contain transfected genes that stably express the proteins shown in parentheses as follows: N223E1 (p53val^{135}). p53val^{135} protein exhibits a wild-type conformation at 32.5°C or a mutant p53 conformation at 37.5°C; it is encoded by plasmid pGp53VS, which carries a copy of the p53val^{135} gene which expresses both wild type and splice variant forms of p53 (Meek and Eckhart, 1990). These cells were maintained at 37.5°C since we found that p53val^{135} allele to be partially wild type at 37°C. Low passage REF52 cells and all cell lines were maintained in Alpha minimum essential media supplemented with 10% fetal bovine serum in a 5% CO_2 atmosphere at 37.5°C, unless otherwise shown.

Cell Cycle Analysis

Cells were seeded and incubated as indicated. Monolayers of cells were washed twice with cold phosphate buffered saline (PBS), trypsinized, and washed twice more with cold TSE (TSE is 100 mM Tris-HCl, 70 mM NaCl, 5 mM EDTA; adjusted to pH 7.5, and filter sterilized). Final cell pellets (1 to 2 × 10^6 cells) were resuspended into 750 µl cold TSE and fixed by drop-wise addition of 2.0 ml of 95% ethanol (-20°C) while gently vortexing, and stored overnight (4°C). Fixed cells were stained for DNA analysis by the propidium iodide method (Krishan, 1975). Briefly, cells were washed twice with cold TSE and then resuspended by gentle vortexing into 1.0 ml of PI Solution (PI Solution is per 100 ml: 25 mg

of propidium iodide (Sigma), 100 mg of sodium citrate, 10 mg of RNase A (Sigma), and 0.1% Triton X 100). Cells were incubated for at least 2 hrs in the dark and then passed through a 41-micron filter (Spectramesh; Spectrum). The cells were analysed by flow cytometry on a Coulter EPICS -Profile IV using Multicycle™ software (Phoenix Flow Systems), which allows for the removal of G1 doublets. 12.5×10^3 to 25×10^3 cells were sampled during each analysis. DNA content was determined by fluorescence and each analysis was normalized to the relative 2N DNA content of the G1 peak. Distribution of the S-phase was determined by a zero order function using Multicycle™ software (Phoenix Flow Systems), and is indicated as a black shaded area on all figures. The scale of the cell number, ordinate axis, is determined by the relative distribution of fluorescent events, maximizing the cell cycle profile.

Centrifugal Elutriation

2.0×10^7 to 5.0×10^7 cells were grown as indicated, taking care that they did not become confluent. Cells were harvested via trypsinization, pelleted by centrifugation and resuspended in elution buffer (PBS + 2.0 μM EDTA). The sample was injected into a Beckman JE-5.0 Elutriation rotor with the standard chamber at 2500 rpm and 40 mls/minute flow rate. 50 ml samples were collected at the initial flow rate and at a reduced rotor speed of 2000 rpm, and after incremental increases of 10 mls/minute in the flow rate until the chamber had emptied (typically at 100 mls/minute flow rate). Resulting samples were pelleted by centrifugation and divided for either flow cytometric analysis or lysed for protein analysis.

Immunoprecipitation and Western Immunoblot Analysis

Cells were lysed for 30 min in 1.0 ml lysis buffer (0.5% Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl [pH 8.0], 50μg aprotinin [Sigma], 5 μM leupeptin, 1 mM PMSF, 10 mM NaF, 50 mM β-glycerophosphate) on ice with intermittent shaking. Cellular debris was removed by centrifugation and the supernatant was precleared by a 2 hour incubation in a - pellet of a 10% suspension of crude *Staphylococcus aureus* (250 μl per sample, washed; Sigma), followed by a 2 hour incubation with 10 μg of murine or rabbit immunoglobulin G2a polyclonal antibody (Sigma) and 50 μl of a 3% suspension of protein A Sepharose beads (preswollen and washed; Pharmacia). Following recentrifugation, 250-500 μg of lysate supernatant was immunoprecipitated overnight with the appropriate antibody 1419F, a Cdc2 rabbit polyclonal antibody (Litchfield et al. 1995). Immune complexes were collected by a subsequent 4 hour incubation with mixing with 100μl of a 3% suspension of protein A Sepharose beads (preswollen and washed; Pharmacia). Eluted samples were loaded onto a 12.5% polyacrylamide gel in sample buffer and in the presence of sodium dodecyl sulphate (SDS), and electrophoresed at 35mA. The gels were processed as indicated below for western blot analysis.

For immunoprecipitations using conjugated antibodies, 4.95 μg/ml Cdc2 specific antibody was incubated with 2 ml preswollen Sepharose A beads (2 mg/ml) for 1 hour at room temperature. The bead slurry was washed two times in 0.2 M sodium borate (ph 9.0), then incubated in 20 mM dimethylpimelimidate for 30 minutes at room temperature. The bead slurry was washed twice for two hours in 0.2M ethanolamine (ph 8.0), then resuspended

in PBS plus 0.01% thimerosal and stored at 4°C until used. Immunoprecipitations were performed as above.

For western immunoblot analysis, 50 μg of lysed samples were loaded as indicated onto a 12.5% polyacrylamide gel in sample buffer and in the presence of SDS, and electrophoresed at 35-40 mA. Protein concentration was determined using the Bio-Rad *DC* Protein assay. The gels were then transfered to nitrocellulose (0.2 μm) for 4 hours at 100V in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). For immunodetection, the membrane was blocked for six to eight hours with either 5% BSA or 5% skim milk powder in TTBS buffer (TBS with 0.1% Tween 20). Primary antibody was added as indicated in 1% BSA in TTBS buffer and incubated overnight. After two washes in TTBS, the secondary antibody (Horseradish Peroxidase conjugate) was added and incubated for 1 hour. The following antibodies were used in this study: 1419F; Phospho-specific Cdc2 (Tyr 15) antibody from New England Biolabs (cat. # 9111S); Cyclin B1 antibody raised against a peptide (aa 391-409) corresponding to the C-terminus of rat Cyclin B1 (Santa Cruz Biotechnology, Inc., cat. # sc-595); Cyclin A (cat. # 05-155) and E (cat. # 06-138) antibodies from Upstate Biotechnology. For Cyclin B1 antibody blocking assay, the antibody was pre-incubated with blocking peptide (cat# sc-327) at a 10 times concentration peptide/antibody for 4 hours at 4°C with periodic shaking. The membrane was developed using the chemiluminescent detection kit from New England Biolabs (cat # 7003).

Cdc2 Kinase Assays:

Cell lysates were collected at the indicated times and conditions and lysed in 1.0%

NP-40, 50mM Tris-Cl, 150mM NaCl, 50 μM aprotinin, 50 mM β-glycerophosphate, 10mM NaF, 0.2M EDTA. Cell lysates were then incubated for 4 hours with 50 μl of a 3% protein A Sepharose and anti-Cdc2 antibody (Litchfield et al. 1995), washed and resuspended in 250 μl kinase buffer (50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT). 18 μl was used for each subsequent reaction. Cdc2 kinase activity was measured at 30°C in a 30 μl reaction mixture containing 50 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM $\gamma[^{32}P]$ ATP (spec. act. 4500 Ci/mMol) and 0.3 mM Cdc2 substrate peptide. Reactions were initiated by the addition of lysate (enzyme) and terminated by spotting 20 μl of the reaction mixture on phosphocellulose P81 paper 30 minutes after initiation as previously described (Litchfield et al. 1995).

Immunofluorescence:

Cells were seeded onto coverslips and grown under the indicated conditions, then fixed in 3.7% formalin for 20-30 minutes at room temperature. The coverslips were washed three times with PBS and stored in PBS at 4°C. For Cyclin B1 staining, the cells were first permeablized in 1%NP-40/PBS for 5 minutes, then blocked with 10% goat serum for 1 hour at 37°C. The slides were washed three times with PBS, then primary antibody (Anti-Cyclin B1 rat specific, Santa Cruz Biotechnology cat# sc-595), or control rabbit IgG added at 1/50 and incubated at room temperature for a minimum of 4 hours. The slides were again washed 3 times with PBS, then incubated for 1 hour with 1/100 anti-rabbit FITC conjugated secondary antibody. The slides were washed 3 times in PBS, then stained with DAPI stain for 5 minutes, washed several times in PBS and mounted onto slides using 2.5% Dabco

(Fluka 33480 1,4 Diazabicyclo(2.2.2)octane) mounting media containing anti-bleach solution (6g Glycerol, 2.4g Mowiol 4-88, 6 ml ddH20, 12ml 0.2M Tris pH 8.5). Analysis was done on a Axiophot microscope (Zeiss) using a CCD photometrics camera and IP lab version 3.1 software (Signal Analytics).

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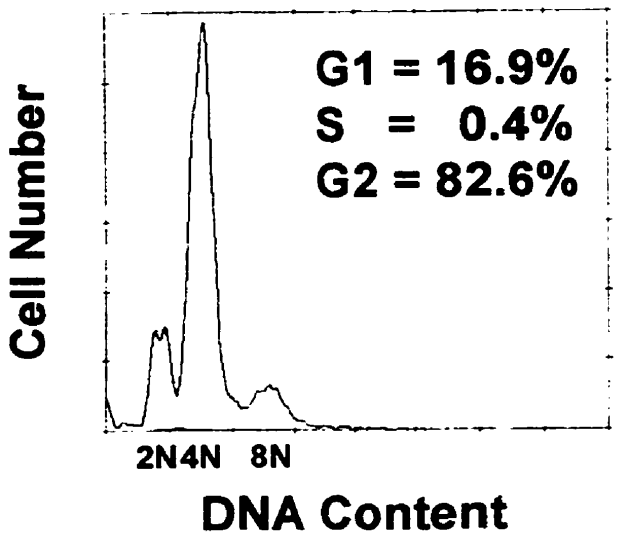
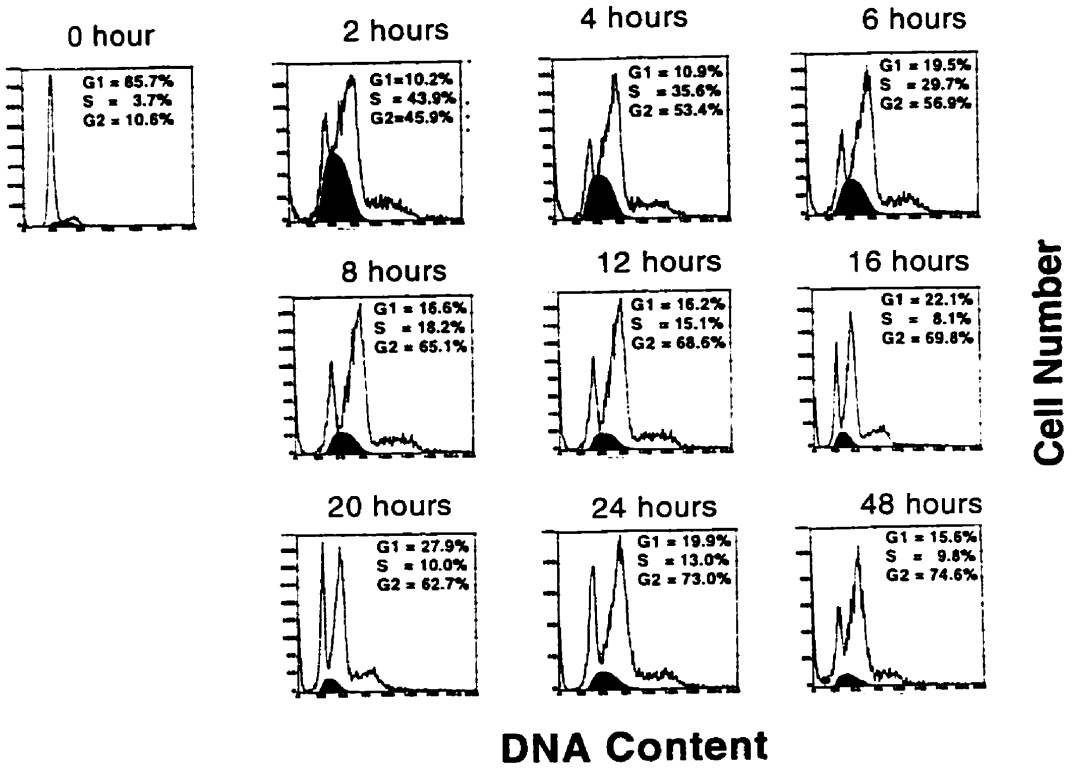
FIGURE 1

Flow cytometric analysis of G2 enriched p53 arrest populations. Panel A: representative example of a purified population of N223E1 cells arrested by p53 at G2 and isolated by centrifugal elutriation as indicated in materials and methods. S phase is indicated as a black shaded area. Panel B: Time course of cell populations released into p53 induced G2 arrest after double synchronization by serum starvation for 24 hours and released into hydroxyurea for 24 hours. The cells were then released from the HU arrest at 32°C, the temperature for wild type p53 formation. Panel C: DAPI staining of cells arrested at G2 by p53 for 48 hours, generated from the double synchronization method.

A



B


0 hour
G1 = 85.7%
S = 3.7%
G2 = 10.6%
2 hours
G1=10.2%
S = 43.9%
G2=45.9%
4 hours
G1 = 10.9%
S = 35.6%
G2 = 53.4%
6 hours
G1 = 19.5%
S = 29.7%
G2 = 56.9%
8 hours
G1 = 16.6%
S = 18.2%
G2 = 65.1%
12 hours
G1 = 16.2%
S = 15.1%
G2 = 68.6%
16 hours
G1 = 22.1%
S = 8.1%
G2 = 69.8%
20 hours
G1 = 27.9%
S = 10.0%
G2 = 62.7%
24 hours
G1 = 19.9%
S = 13.0%
G2 = 73.0%
48 hours
G1 = 15.6%
S = 9.8%
G2 = 74.6%
Cell Number
DNA Content

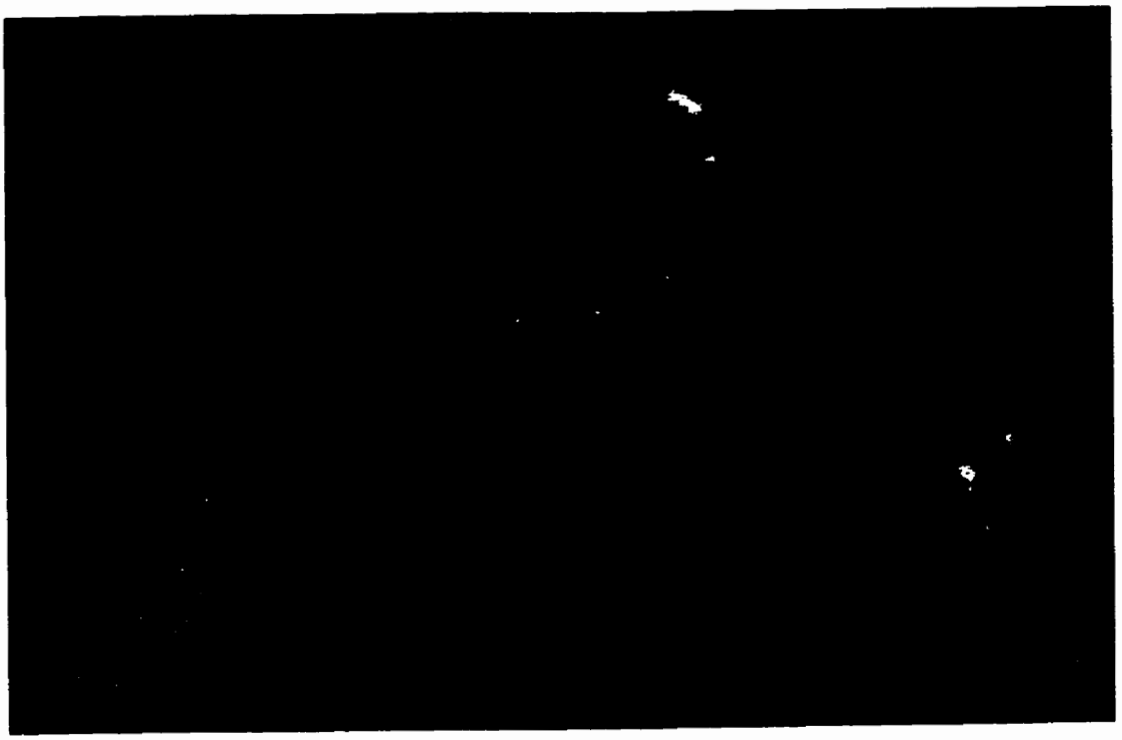


FIGURE 2

Cdc2 kinase activity in p53 blocked REF52 cells. Cdc2 kinase activity was determined by an immunoprecipitation/kinase assay as described in the materials and methods. The IgG control is rabbit IgG. Cells were synchronized prior to the p53 G2 block by 1) serum starvation for 24 hours, then 2) released with serum and arrested in early S phase with hydroxyurea for a further 24 hours. The cells were placed at the non-permissive temperature for a further 8 hours prior to the removal of hydroxyurea. Cells arrested at the p53 G1 block were first synchronized by serum starvation for 24 hours, then released with serum and transfered to the non-permissive temperature. Controls for cdc2 kinase treatment were done in REF52 cells at the non-permissive temperature. Cells were arrested for 24 hour (hydroxyurea) or overnight (nocodozole) respectively. Shown is the standard error of the mean for five independent experiments.

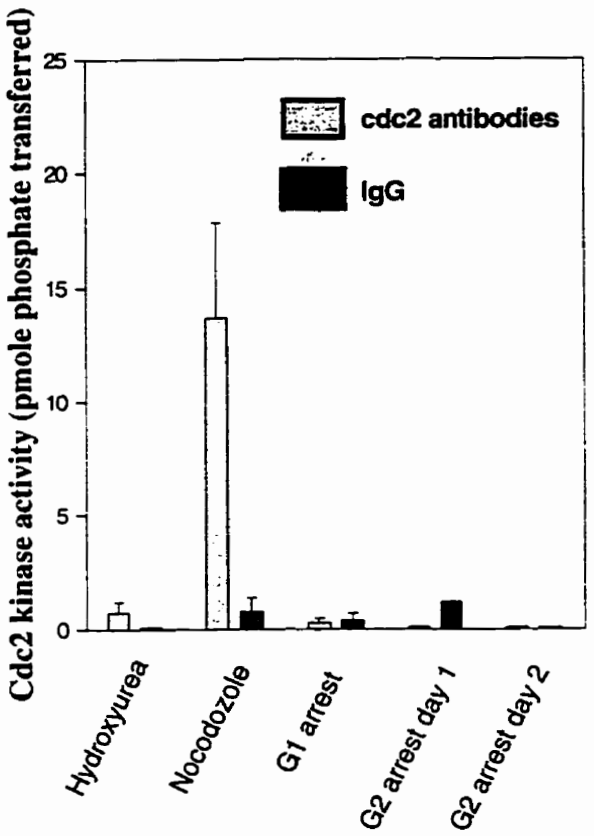

Cdc2 kinase activity (pmole phosphate transferred)
25
20
15
10
5
0
cdc2 antibodies
IgG
Hydroxyurea
Nocodozole
G1 arrest
G2 arrest day 1
G2 arrest day 2

FIGURE 3

Cdc2 protein expression in cycling and p53 blocked REF52 cells. REF52 cycling cells were separated by centrifugal elutriation. N223E1 p53 blocked cells were first synchronized in S phase with hydroxyurea (HU) and shifted to 32°C to induce wild type p53 before HU removal. Forty-eight hours after temperature shift cells were separated by centrifugal elutriation as previously indicated. Cell extracts from elutriation fractions were western immunoblotted using anti-Cdc2 antibodies and their positions in the cell cycle determined by flow cytometry.

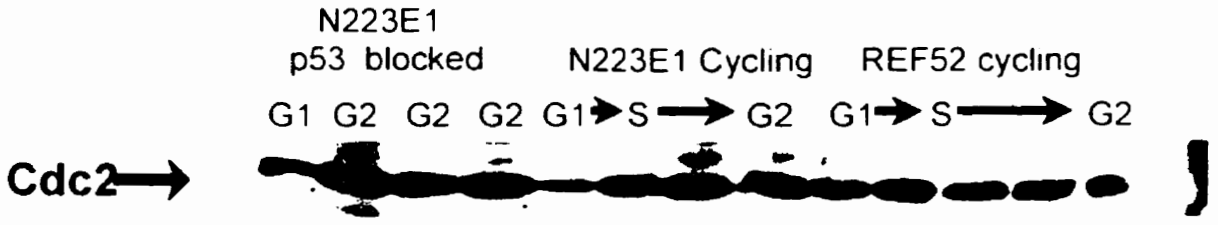
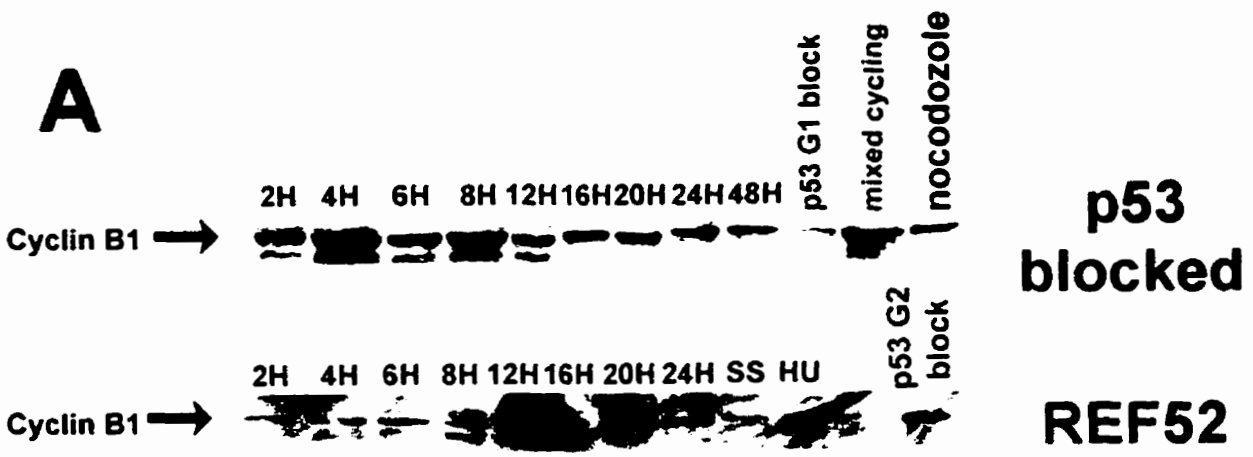

N223E1
p53 blocked
N223E1 Cycling
REF52 cycling
G1 G2 G2 G2 G1 S G2 G1 S G2
Cdc2

FIGURE 4

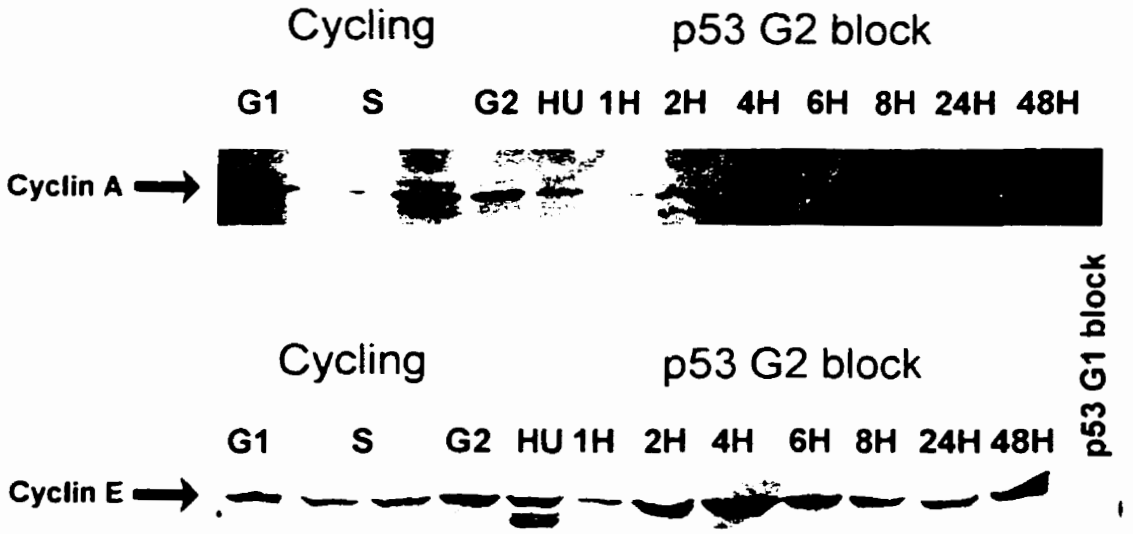
Cyclin Expression in p53 blocked N223E1 cells: A: Cyclin B1 protein expression. N223E1 cells were arrested 24 hours with hydroxyurea, shifted to 32°C for a minimum eight hours to induce wild type p53 before release and then isolated at various points into G2 arrest. The control REF52 cells were treated in an identical manner. p53 G1 blocked cells were arrested for 24 hours by serum starvation, then shifted to 32°C with serum addition for 48 hours. Proteins were separated by SDS-PAGE, transfered and probed with anti-Cyclin B1 antibody. Top panel: Arrested cell line populations Bottom panel: cycling REF52 cells. (HU - hydroxyurea treated SS - serum starved cells)

B: Cyclin A and E protein expression. Cells lysates were collected at various times into G2 arrest as previously described and compared to lysates from cycling cells separated by centrifugal elutriation. Western blots were probed with either anti-Cyclin A (top) or anti-Cyclin E (bottom) specific antibodies.

C: Cyclin B1 association with Cdc2 kinase. Cell lysates were collected at various times into G2 arrest as previously indicated and immunoprecipitated with Cdc2 antibody conjugated to Sepharose A, separated on SDS-PAGE and transferred. Top panel: probed with anti-Cyclin B1 and anti-Cdc2 antibodies. Bottom panel: The same gel, stripped with 5% mercaptoethanol at 56°C and reprobed with anti-Cyclin B1 antibodies blocked with peptide, and anti-Cdc2 antibodies. (NC - nocodozole treated G1 - p53 arrested cells)


A
2H 4H 6H 8H 12H 16H 20H 24H 48H
p53 G1 block
mixed cycling
nocodozole
Cyclin B1
p53 blocked
2H 4H 6H 8H 12H 16H 20H 24H SS HU
p53 G2 block
Cyclin B1
REF52

B


Cycling
p53 G2 block
G1
S
G2
HU
1H
2H
4H
6H
8H
24H
48H
Cyclin A
p53 G1 block
Cycling
p53 G2 block
G1
S
G2
HU
1H
2H
4H
6H
8H
24H
48H
Cyclin E

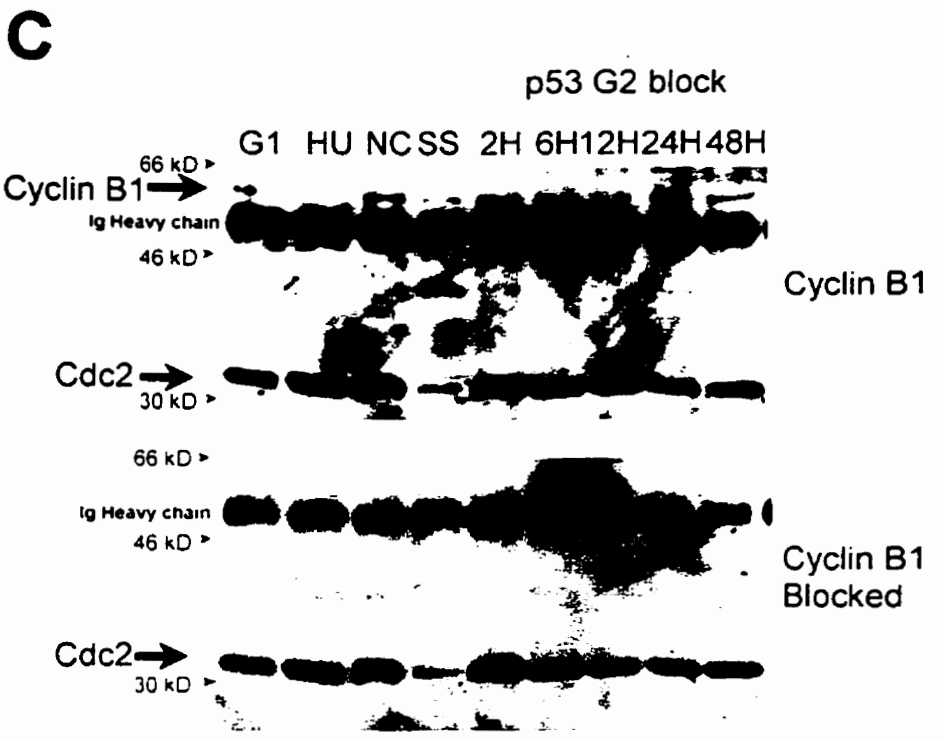

C
p53 G2 block
G1 HU NC SS 2H 6H 12H 24H 48H
66 kD
Cyclin B1
Ig Heavy chain
46 kD
Cyclin B1
Cdc2
30 kD
66 kD
Ig Heavy chain
46 kD
Cyclin B1
Blocked
Cdc2
30 kD

FIGURE 5

Phosphorylation status of Cdc2 kinase in p53 blocked cells. Cell lysates were obtained from N223E1 cells at various times into p53 induced G2 arrest and from companion non-arrested REF52 cells, as well as from control populations (p53 G1 blocked and mixed cycling) as previously indicated. Proteins were precipitated with anti-Cdc2 kinase antibodies and separated by SDS-PAGE, transferred and probed with either anti-Cdc2, or anti-Cdc2 phosphotyrosine 15 antibodies. Panel A: N223E1 arrested cell precipitates. Panel B: REF52 cell precipitates after HU release. (SS- Serum Starved HU- hydroxyurea NC- nocodozole treated cells)

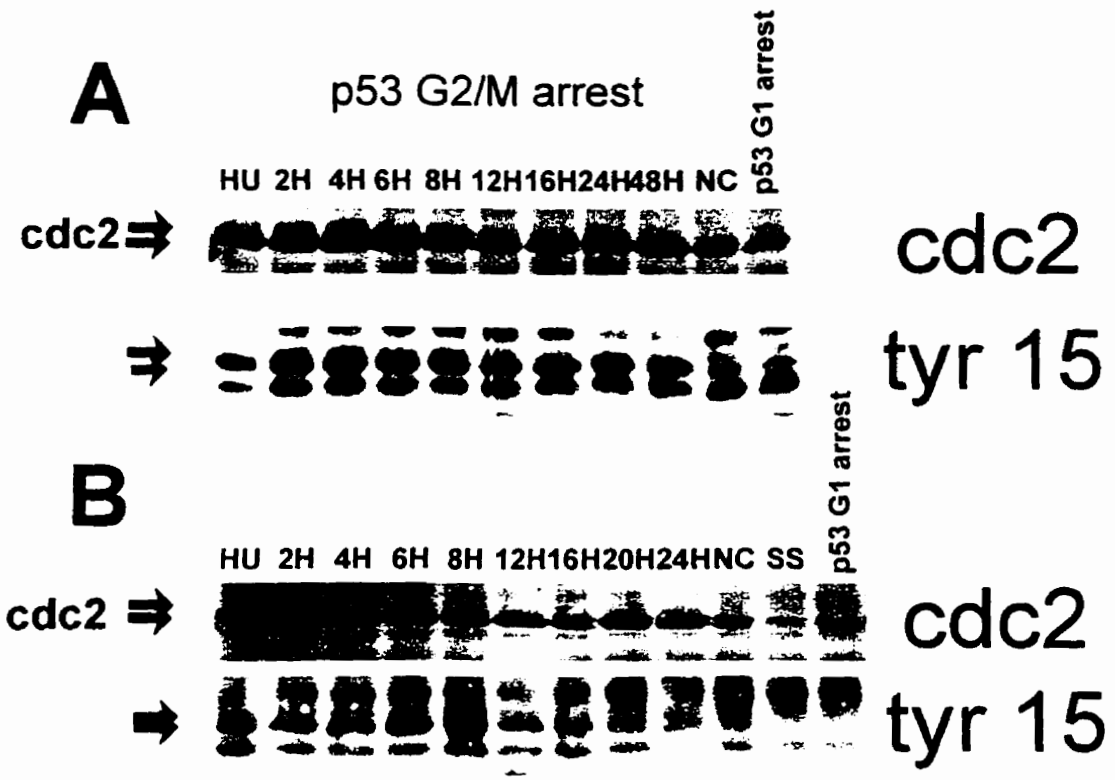
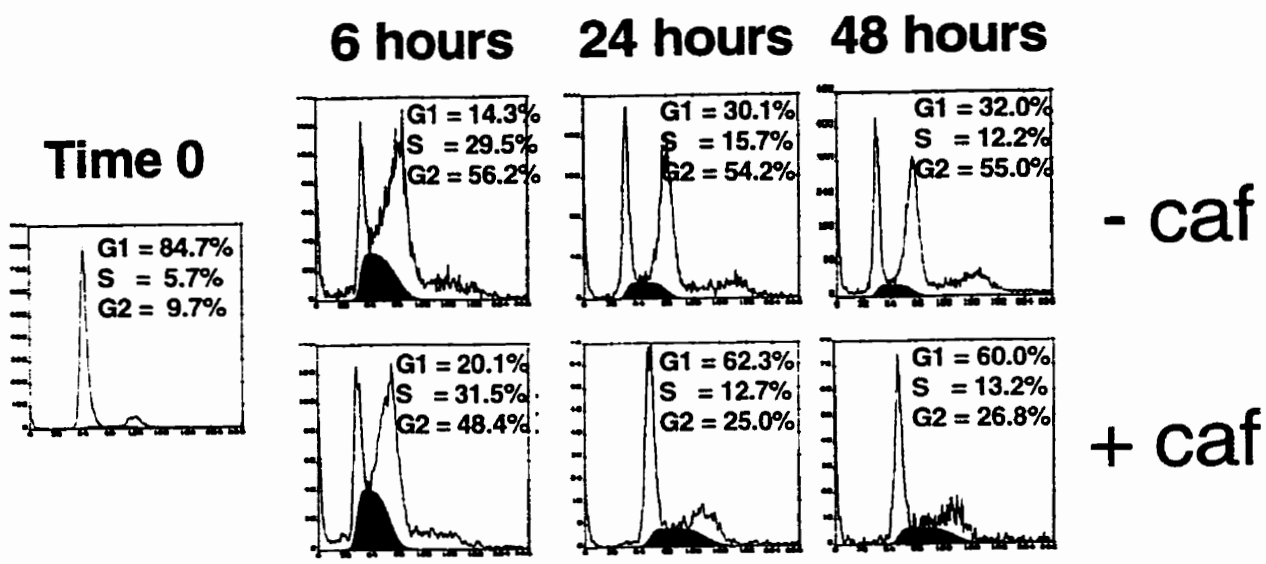

A
p53 G2/M arrest
HU 2H 4H 6H 8H 12H16H24H48H NC
p53 G1 arrest
cdc2
cdc2
tyr 15
B
HU 2H 4H 6H 8H 12H16H20H24HNC SS
p53 G1 arrest
cdc2
cdc2
tyr 15

FIGURE 6

Caffeine overcomes p53 G2 block. A: N223E1 cells were first synchronized by hydroxyurea as previously indicated (Time 0), then released at 32°C and maintained either 8-12 hours with or without 2 mM caffeine. The media was then changed and the cells collected for DNA flow cytometric analysis at the times indicated. B: Growth curves of N223E1 cells after caffeine treatment. 1 x 10^5 N223E1 cells were seeded onto 60 mm dishes and treated as above, and their growth compared to control N223E1 cells grown at the permissive temperature (37.5°C) and parental REF52 cells grown at 32°C. Cells were trypsinized and whole cell populations counted on a haemocytometer. Shown is the standard error of the mean for four independent experiments.


A
6 hours
24 hours
48 hours
Time 0
G1 = 84.7%
S = 5.7%
G2 = 9.7%
G1 = 14.3%
S = 29.5%
G2 = 56.2%
G1 = 30.1%
S = 15.7%
G2 = 54.2%
G1 = 32.0%
S = 12.2%
G2 = 55.0%
- caf
G1 = 20.1%
S = 31.5%
G2 = 48.4%
G1 = 62.3%
S = 12.7%
G2 = 25.0%
G1 = 60.0%
S = 13.2%
G2 = 26.8%
+ caf

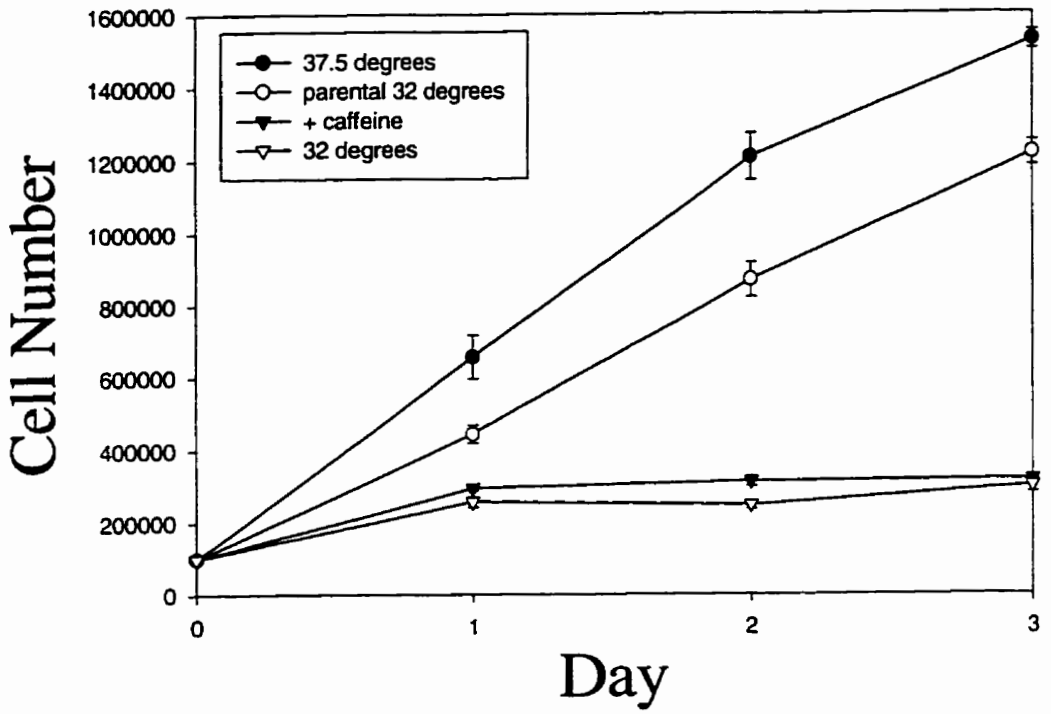
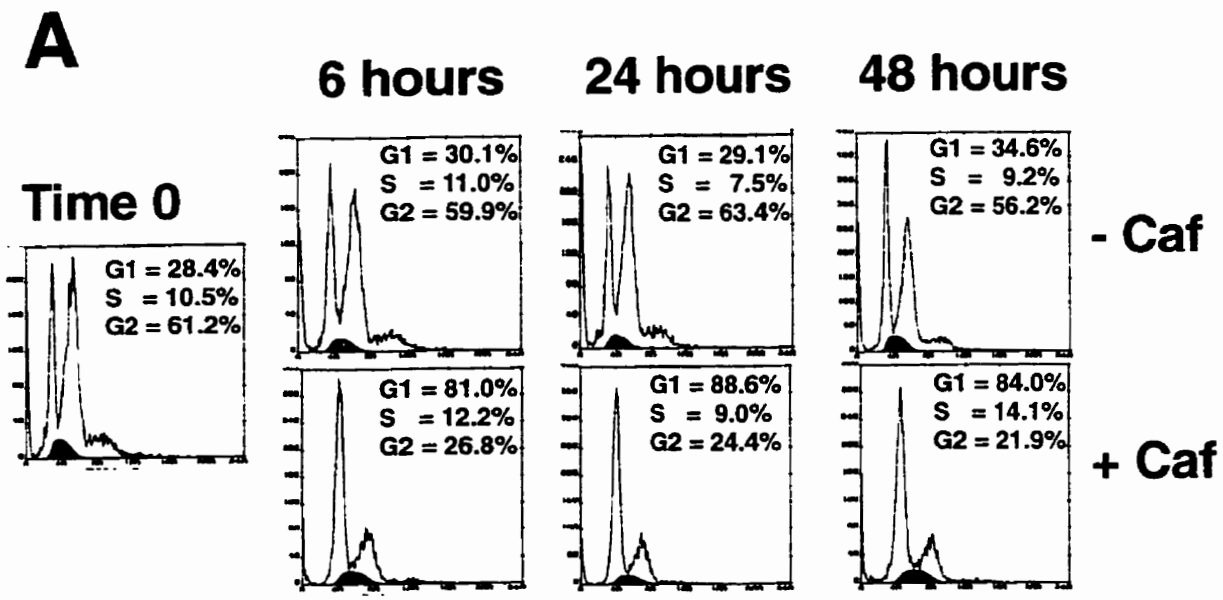

Cell Number
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
0
1
2
3
Day
37.5 degrees
parental 32 degrees
+ caffeine
32 degrees

FIGURE 7

Effect of caffeine addition on established p53 G2 blocked REF52 cells. A: N223E1 cells prepared as in Figure 6 were allowed to arrest at G2 for 24 hours (Time 0). They were then treated for 4-6 hours with 2 mM caffeine, placed in fresh media and collected at the times indicated. Control N223E1cells did not receive any caffeine. B: Addition of caffeine does not release p53 G1 blocked cells. N223E1 cells were synchronized by serum starvation then allowed to arrest at G1 for 24 hours at 32°C (Time 0). They were then treated for 6 hours with 2 mM caffeine, placed in fresh media and collected at the times indicated.


A
6 hours
24 hours
48 hours
Time 0
G1 = 28.4%
S = 10.5%
G2 = 61.2%
G1 = 30.1%
S = 11.0%
G2 = 59.9%
G1 = 29.1%
S = 7.5%
G2 = 63.4%
G1 = 34.6%
S = 9.2%
G2 = 56.2%
- Caf
G1 = 81.0%
S = 12.2%
G2 = 26.8%
G1 = 88.6%
S = 9.0%
G2 = 24.4%
G1 = 84.0%
S = 14.1%
G2 = 21.9%
+ Caf

B

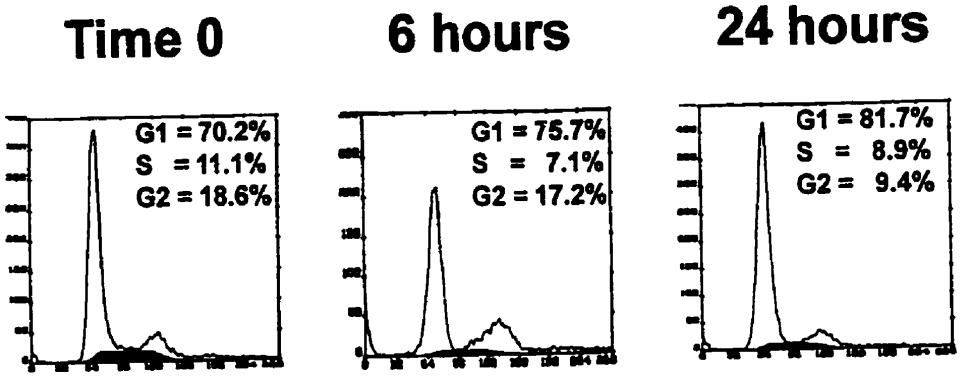

Time 0
G1 = 70.2%
S = 11.1%
G2 = 18.6%
6 hours
G1 = 75.7%
S = 7.1%
G2 = 17.2%
24 hours
G1 = 81.7%
S = 8.9%
G2 = 9.4%

FIGURE 8

Cdc25C protein expression in p53 G2 blocked cells. Cell lysates were obtained at various time points: N223E1 after p53 induced G2 arrest, companion non-arrested REF52 cells, and cells prevented from arresting at G2 by caffeine addition as previously indicated. Protein extracts were separated by SDS-PAGE, transferred and probed with anti-Cdc25C antibody. Arrows indicate the three forms of Cdc25C observed.

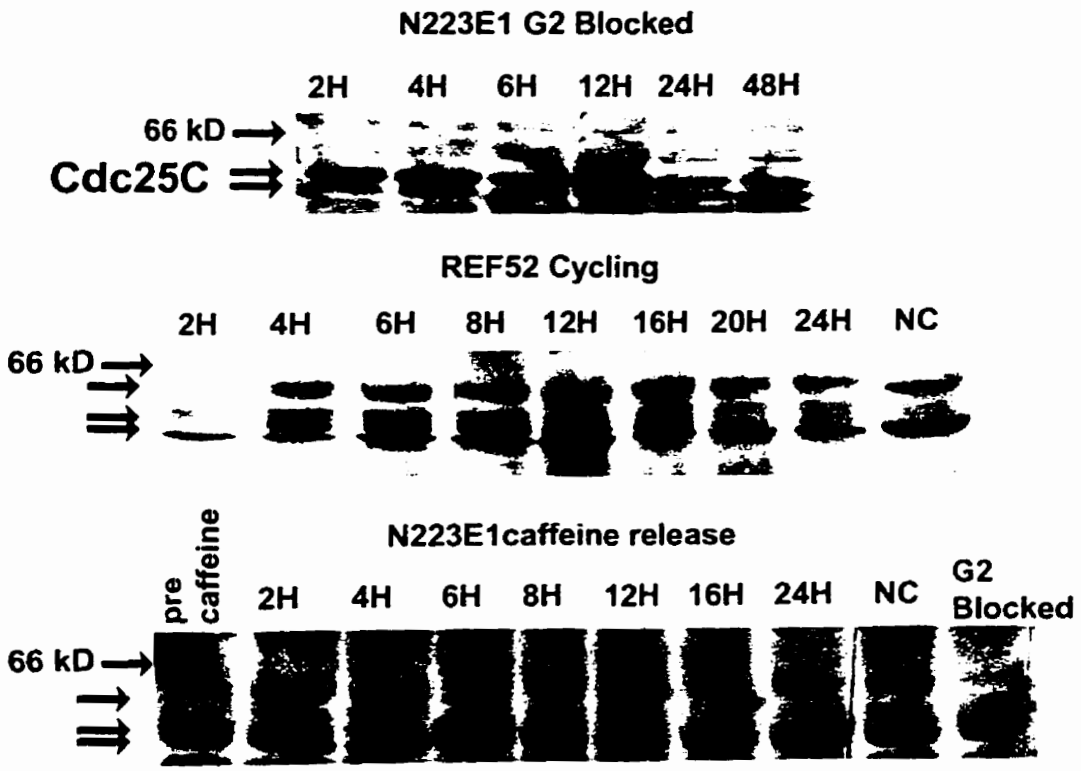
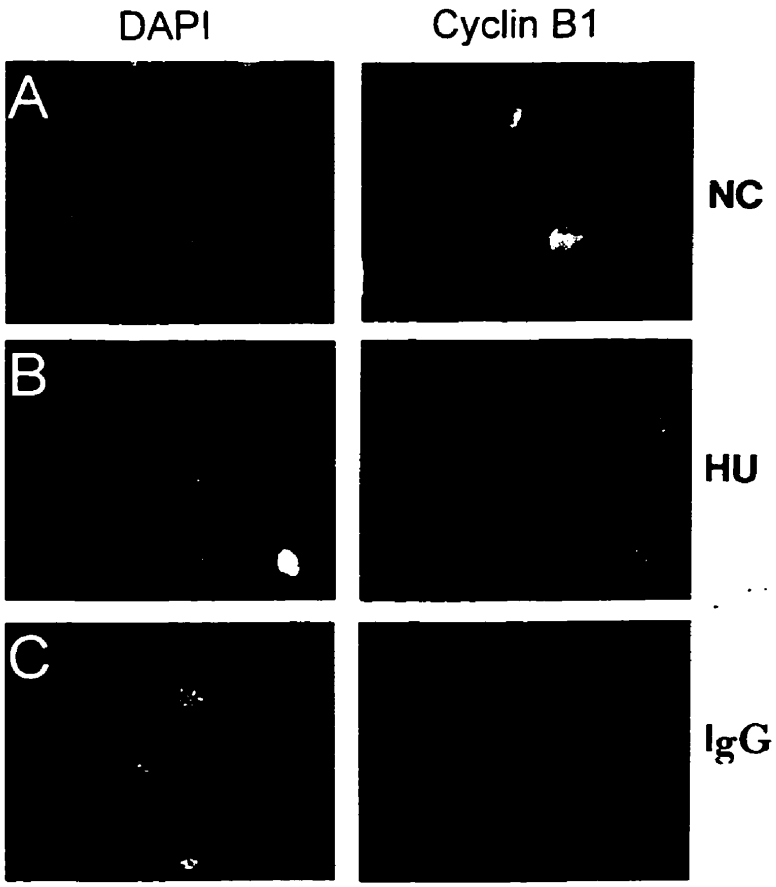
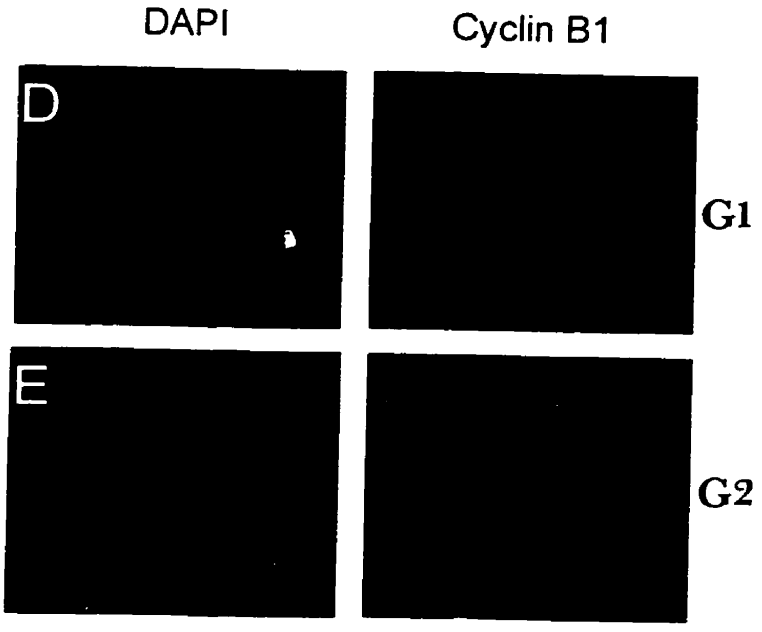

N223E1 G2 Blocked
2H
4H
6H
12H
24H
48H
66 kD
Cdc25C
REF52 Cycling
2H
4H
6H
8H
12H
16H
20H
24H
NC
66 kD
N223E1caffeine release
pre caffeine
2H
4H
6H
8H
12H
16H
24H
NC
G2 Blocked
66 kD

FIGURE 9

Cyclin B1 expression in G2 arrested cells. N223E1 cells were seeded onto coverslips and treated as follows. Control cells were arrested with either nocodazole (A) or hydroxyurea (B). Alternatively, cells were arrested for 24 hours by p53 at G1 (D) or G2 (E). The coverslips were collected and stained for Cyclin B1 expression (A,B,D,E, right panel) and with DAPI for nuclear content (left panel) as indicated in materials and methods. G2 arrested cells stained with control antibody (rabbit IgG) in place of Cyclin B1 antibody as a negative control are shown in (C). (HU - hydroxyurea NC- nocodazole treated cells; G1 - p53 G1 arrested G2 - p53 G2 arrested cells)


DAPI
Cyclin B1
A
NC
B
HU
C
IgG


DAPI
Cyclin B1
D
G1
E
G2

Discussion and Future Directions

Using REF52 (rat embryo fibroblast) cell lines expressing a temperature sensitive val135 p53 mutant (Finlay et al. 1988) in this work, I demonstrated that p53 can induce cell cycle arrest at G2/M (Stewart et al. 1995). Parallel observations have been made by several other groups (Guillouf et al. 1995; Vikhanskaya et al. 1994; Agarwal et al. 1995; Aloni Grinstein et al. 1995). Vikhanskaya et al (94) transfected a human ovarian cancer cell line which does not express p53 with the same p53val^{135} mutant. Similar to my cells, their cell lines accumulated at G2/M (ibid). Interestingly they did not arrest at G1, suggesting that some changes such as loss of p21$^{waf/cip1}$ may have occured to these cells prior to the readdition of p53, although the authors did not check this possibility. They did note Gadd45 mRNA was stimulated. However whether there was a corresponding increase in protein levels was not determined. Akin to this, Agarwal et al (95) reported G2/M arrest in human Li-Fraumeni fibroblasts which had human p53 restored using a plasmid with the tetracyline repressor system. They did note an increase in p21$^{waf1/cip1}$ levels in arrested cells, however they were using mixed G1/G2 arrested populations and did not test the G2 cells on their own. In both of these papers the arrest at G2 was reversible, similar to what has been observed in our laboratory.

The results from the two other groups, however, point to the complexity of p53 induced G2 checkpoint control. This highlights the differences seen in p53 function depending on cell types and backgrounds. Aloni-Grinstein et al (95) also reported the presence of a p53 dependent G2 block in the 70Z/3 pre-B cell line subject to γ-irradiation,

which may be related to B-cell differentiation (Aloni-Grinstein et al. 1995). The same authors had previously noted that upregulation of p53 expression was associated with pre-B cell differentiation (Aloni-Grinstein et al. 1993). γ-irradiation of the 70Z/3 pre-B cell line resulted in increased p53 protein levels and both apoptosis and differentiation. Arrest at G1 was associated with the induction of apoptosis, whereas the majority of cells which expressed a marker for B cell differentiation (κ light chain) had arrested at G2 (ibid). $P21^{waf1/cip1}$ expression was primarily restricted to the G1 population, suggesting that it probably does not have a role in G2 arrest (ibid). Mutant p53 blocks wt induced κ light chain gene expression and interfered with arrest at G2 and the induction of differentiation. G2 delays are associated with both differentiation and development in a number of cells types (for review, see Mowat and Stewart, 1998). It may be that p53's ability to arrest at G2 is employed by cells in specific circumstances to allow for differentiation.

In contrast to the above, the addition of the $p53val^{135}$ mutant to M1 myeloblastic leukemia cells increased exit from G2 arrest in response to radiation, which correlated with p53-induced apoptosis (Guillouf et al. 1995). This occured whether $p53val^{135}$ was in mutant or wild type conformation (ibid). Cells which arrested at G2 longer were better able to resist apoptosis, while conversely the quicker cells left G2 the better they apoptosed (ibid). It should be noted that these cells differ from the cells mentioned above in that they still cycle in the presence of wtp53. This suggests at least some of the arrest pathways employed by wtp53 may be altered in the M1 cells. It also emphasises the difference in p53 action in different cell lines based on their genetic background.

Why do I not observe Apoptosis in the REF52 cells?

I found no evidence for apoptosis in REF52 cells when wild type p53 is over expressed with or without the *ras* oncogene (unpublished data). Previous work had indicated that apoptosis induced by the adenovirus E1A oncogene in REF52 cells is dependent on wild type p53 (Lowe and Ruley, 1993). This may indicate that over expression or stabilization of p53 alone is insufficient to induce apoptosis in REF52 cells, and instead needs the presence of another oncogene such as E1A. Consistent with this proposal is that fibroblasts expressing the E1A gene are more prone to ionizing radiation and chemotherapeutic drug induced apoptosis (Lowe et al. 1993). Conversely E1A expressing p53 negative fibroblasts or untransfected normal fibroblasts show little apoptosis (ibid). Human ovarian cancer cell lines transfected with the same temperature sensitive mutant also did not show evidence of apoptosis even after 72 hours at the restrictive temperature (Vikhanskaya et al. 1994). Along these lines, NIH3T3 cells transformed by either oncogenic Src, Ras, or Raf are more sensitive to etoposide induced apoptosis than the parentals (Chen et al. 1997). These cell lines exhibited induction of p21 (but not p53) (ibid). Thus, the inability to induce apoptosis in these cell lines is not unexpected, most likely reflecting the genetic background of the cells.

What About Mitotic Catastrophe?

A second possibility is that release of my cells from p53 induced G2 arrest by the addition of a drug such as caffeine may force them into mitosis prematurely, causing them to undergo a different destructive event termed mitotic catastrophe. It is essential that

mitosis is initiated only after sufficient cell growth and DNA replication have been completed (reviewed in Forsburg and Nurse, 1991). Mitotic catastrophe occurs when a cell attempts to prematurely enter mitosis, initiating breakdown of the nuclear envelope and chromosome condensation but are unable to divide and survive (ibid). Induction of mitotic catastrophe in many cases centres around regulation of the cell cycle proteins. Mitotic catastrophe can result from the inactivation of both Mik1 and Wee1 kinases (Russell and Nurse, 1987; Lundgren et al. 1991). Mutation of Tyr 15 (Cdc2-Y15F) in *S. pombe* results in mitotic catastrophe in 10% of cells per generation (Gould and Nurse, 1989; Sorger and Murray, 1992). Further, cotransfection into HeLa of Cyclin B1-Cdc25C, Cyclin B1-Cdc2 or Cyclin A-Cdk2 caused mitotic catastrophe in 48-65% of cells, which could be ablated by the addition of Wee1 (Heald et al. 1993). It is evident though that more than just Tyr 15 phosphorylation is required to regulate mitotic timing. Transfection of a double phosphorylation site mutant (A14, F15) gave only 12% mitotic catastrophe (Heald et al. 1993). Cotransfection with Cyclin A or B resulted in a dramatic increase (75-95%) of cells mitotic catastrophe (ibid). Thus, endogenous control over cyclin expression may prevent the action of the Cdc2 regulation resistant mutant in promoting mitotic catastrophe.

Addition of caffeine can abrogate G2 arrest in response to irradiation in p53 null cells and sensitize them to irradiation induced apoptosis (Yao et al. 1996; DeFrank et al. 1996). However, addition of caffeine is not always associated with mitotic catastrophe (Yao et al. 1996; DeFrank et al. 1996). I have no evidence for the occurrence of mitotic catastrophe in the N223E1 cells, when they are released from G2 arrest by caffeine. This might suggest that the cells are arrested close enough to the G2/M boundary that they can survive release

into mitosis. It may also be that some of the same mechanisms which render cells resistant to apoptosis may also provide resistance to mitotic catastrophe. I kept the dosage of caffeine at the minimum concentration and length of exposure required to induce release. It may be at a higher concentration mitotic catastrophe is induced, if it promotes an accelerated progression through G2 and mitosis. One question, however, is whether these cells would be able to release from the subsequent G1 arrest if they were relieved of wtp53 expression.

Cdc2 expression during G2 arrest.

My efforts to further characterize the mechanism(s) involved in p53's induction of the G2 block were concentrated on Cdc2 kinase. Cdc2 kinase was inactive in G2 arrested cells. The level of Cdc2 protein expression remained unchanged in both G1 and G2 arrested populations, compared to normal cycling cells in the same position of the cell cycle. This implies a different mechanism than what may be in play during quiescence-early G1. A recent report suggests that p53 is required for Cdc2 protein/mRNA downregulation after ionizing radiation of fibroblasts in early G1 (Azzam et al. 1997). This downregulation also requires the presence of $p21^{waf1/cip1}$ protein (ibid), suggesting that it was not a direct transrepressional event by p53 protein on the Cdc2 gene. It should also be noted that this downregulation occurred at least several hours past the induction of p53 expression, which might not be as effective a mechanism at G2, where Cdc2 protein would have to be swiftly removed to prevent progression into mitosis. This does not exclude the possibility that downregulation of Cdc2 kinase expression might be more important later into arrest at G2 for maintenance. However I did not observe any significant decrease in Cdc2 protein

expression for up to two days into G2 arrest.

From my studies on Cdc2 kinase, I concluded that p53 induces arrest at G2 by altering the regulatory events allowing for dephosphorylation and activation of Cdc2 kinase, preventing progression into mitosis and completion of the cell cycle. Cdc25C, the phosphatase which acts upon Cdc2 to dephosphorylate tyrosine 15 and activate Cdc2 may not be phosphorylated (which is required for Cdc25C activation) in the G2 arrested population, suggesting one possible mechanism for p53 inactivation. Release from G2 arrest by caffeine results in the appearance of the phosphorylated band associated with activation. There was also the loss of the inhibitory Ser216 band later into the caffeine release.

The possibilities from this research are many. Control over Cdc2 phosphorylation and activity occurs at a number of levels, thus it will require careful effort to determine which pathways are more important for p53 action. I will attempt to address the more likely scenarios based on my results.

What role does phosphorylation have in p53 arrest?

My results suggest that for initiation of arrest at G2, Cdc2 is maintained in an inactive phosphorylated state at least at Tyr 15. The nature of my experiments did not allow me to determine if Thr 14 is also phosphorylated, although the appearance of an upper band in my arresting cells is suggestive of there being triple phosphorylation (Chapter 3, Figure 5). This may be as important an event for maintaining Cdc2 kinase in an inactive state as tyrosine phosphorylation. This might be addressed by performing phosphopeptide mapping on the

Cdc2 kinase protein from my arrested populations to look for phosphorylation on Thr 14 (Krek and Nigg, 1991; Boyle et al. 1991). While ideally a phospho-specific antibody raised against Thr 14 could be used in a similar set of experiments as Tyr 15, to my knowledge no such antibody is currently available. This phosphorylation event is regulated by Myt1 kinase (Liu et al. 1997; Booher et al. 1997) thus the status of the Myt1 protein should be checked for both levels and activity.

These results still take an indirect approach at determining phosphorylation's role in p53 arrest at G2. A more direct approach would be to overcome the normal controls over Cdc2 kinase at G2 and observe what happens if we attempt to arrest the cells at G2. Our lab has obtained a phosphorylation site mutant (Cdc2AF) from Dr. D.O. Morgan, which has had Thr 14 changed to Ala and Tyr 15 to Phe, rendering the protein constitutively active when bound to cyclin (Jin et al. 1996). The gene for this mutant is contained on a plasmid with the tet promoter, allowing for selective induction (ibid). This mutant has been previously shown to overcome the G2 irradiation checkpoint, but not the earlier S phase arrest (ibid). CdcAF is being placed into the parental REF52 cells along with the temperature sensitive p53 mutant I used. Similar to my experiments, the cells can be synchronized at the G2 arrest and then Cdc2AF induced by the removal of tetracyclin. The control would be a plasmid with wild type Cdc2 under the same inducible promoter, which is still subject to normal cell cycle control (Jin et al. 1996). The prediction is that this mutant should overcome the p53 induced arrest and propel the cells through mitosis and into G1, where they should be arrested by the p53 induced G1 checkpoint.

If they do not arrest, it might suggest that there is a cdk inhibitor at work,

phosphorylation being only a secondary effect of the arrest. As mentioned in the introduction, there is evidence in other species of potential Cdc2 inhibitors, although to this date none have been identified in humans. This inhibitor is unlikely to be $p21^{waf1/cip1}$ since cells from knockout mice still maintain their G2 arrest point (Deng et al. 1995; Brugarolas et al. 1995). I had noted in some initial ^{35}S immunoprecipitations of Cdc2 kinase in both p53 induced G1 and G2 arrested cells that there was the appearance of an approximately 39 kD band coimmunoprecipitating with Cdc2 kinase in my arrested populations (unpublished data). It should be noted that these cells had been arrested for a minimum of 48 hours before the immunopreciptations were done. It is possible that the mode of action for the arrest may change later on, where induction of a Cdc2 inhibitor might be more important than phosphorylation for long term inhibition. Whether this could be a Cdc2 kinase inhibitor remains to be determined, although efforts are underway in the lab to isolate and identify the protein.

What role does Cdc25C and its regulation have on G2 arrest?

Presuming successful results from the above experiments on Cdc2 phosphorylation, we need to look more thoroughly at the regulator proteins involved. Control over Cdc2 phosphorylation could be happening through Cdc25C or Wee1/Myt1. My initial results suggest Cdc25C may be inactive in the G2 arrested populations, as the protein remains hypophosphorylated. Cdc25C has been implicated in control over DNA damage induced G2/M arrest previously (O'Connell et al. 1997; Al-Khodairy et al. 1994; Walworth and Bernards, 1996; Hoffman et al. 1993). G2 delay induced by low doses of UV irradiation

results in accumulation of inactive Cdc2/Cyclin B1 (Herzinger et al. 1995; Poon et al. 1996; Gabrielli et al. 1997), and hypophosphorylation of Cdc25C (Gabrielli et al. 1997), similar to what I observed in p53 G2 arrest. This arrest could be relieved by Cdc25C addition *in vitro* (Herzinger et al. 1995).

p53 may be maintaining Cdc2 in the inactive state by influencing one of the proteins which regulate Cdc25C. Cdc25C is regulated differentially by three proteins: negatively regulated by Chk1 kinase which phosphorylates at serine-216, positively regulated by the polo like kinase Plx1, and by PP2A, which may also maintain Cdc25C in its inactive state. The first possibility is p53 works at inducing the inhibitory Serine 216 phosphorylation. In the arrested cells used in this study, I see a single band above the non-phosphorylated Cdc25C band on protein gels consistent with phosphorylation at Serine 216. This band appears to be strongest early in arrest, and may diminish somewhat the longer the cells remain at G2, suggesting this event might be more important for initiation of arrest by p53. Chk1 kinase has been implicated in mediation of radiation induced G2/M arrest (O'Connell et al. 1997; Walworth et al. 1993; Al-Khodairy et al. 1994; Walworth and Bernards, 1996). A recent report suggests that p53 may employ 14-3-3σ, working in cooperation with Chk1 to induce arrest at G2 (Hermeking et al. 1997; reviewed in Nurse, 1997). The 14-3-3σ gene in human colorectal cells is upregulated in response to irradiation, and its product induces an arrest at G2 prior to mitosis when overexpressed (Hermeking et al. 1997). This upregulation is mediated by wild type p53, and does not occur if the cells lack functional p53 protein (ibid). Binding of 14-3-3σ to Cdc25C may block Cdc25C function by sequestering the protein away from its target (ie. Cdc2/Cyclin B1) (ibid; (Peng et al. 1997; Nurse, 1997)).

This possibility will be discussed in a later section. Importantly, Cdc25C must be phosphorylated at Serine 216 to bind effectively with 14-3-3 (Peng et al. 1997), which suggests that the action of both proteins is required for arrest. It should be noted that in the colorectal cancer cell system used to study 14-3-3σ and p53 arrest, in cycling cells Cdc25C was always phosphorylated, which may account for why 14-3-3σ overexpression alone could arrest the cells at G2 (Hermeking et al. 1997). Thus, the role Chk1 kinase has in p53 induced G2 response needs to be determined. The presence of active Chk1 kinase may also be important for Wee1 function, which will be discussed later.

A second possibility is that p53 is functioning by preventing the activation step of Cdc25C. When I released my cells from G2 arrest with caffeine I noted the appearance of a phosphorylated higher molecular weight band which is associated with active protein, and gradual loss of the second single phosphorylation band. Plk1 is one kinase which binds to and activates by phosphorylation of Cdc25C (Kumagai and Dunphy, 1996; Lane and Nigg, 1996). Plk1 is related to the Polo family of protein kinases (ibid), which are required for progression through mitosis ((Llamazares et al. 1991); (reviewed in (Glover et al. 1996)). The protein binds to the mitotic spindle apparatus (Golsteyn et al. 1995), and may be involved both in centrosome maturation and checkpoint control (Lane and Nigg, 1996). Injection of anti-Plk1 antibodies into HeLa cells disrupted chromatin condensation, and dramatically reduced the size of the centrosomes and the amount of gamma tubulin present (ibid). Control over Plk1 activity by p53 could then have a number of complementary effects. By preventing its activation of Cdc25C, it could keep Cdc2 inactive and delay entry into mitosis. In addition, p53 might employ Plk1 kinase to control centrosome duplication and

thus mitosis. Loss of p53 is associated with abnormal amplification of centrosomes (Fukasawa et al. 1996). It would be interesting to determine the status of Plk1 in the cells described in that paper. Plk1 kinase activity and protein levels could be examined in my G2 arrested cells. A protocol for a Plk1 kinase assay exists in the literature (Golsteyn et al. 1995). I might anticipate that kinase activity is reduced or missing since Cdc25C does not show the upper band associated with mitotic activity in G2 arrested cells.

Alternatively, p53 might prevent phosphorylation and activation of Cdc25C by either directly or indirectly affecting PP2A function. PP2A acts as an inhibitor of Cdc25C activity, maintaining it in an underphosphorylated state. Binding of PP2A by Hox11 (a homeobox gene) decreased PP2A activity and prevented γ-irradiation induced G2 arrest in a T-cell line (Kawabe et al. 1997). Microinjection of Hox11 also promoted progression into mitosis of *Xenopus* oocytes arrested at G2 (ibid). This may indicate a role for PP2A in G2 arrest, however Hox11 also binds and inhibits PP1, making the interpretation difficult. PP2A can be inhibited by the addition of drugs such as okadaic acid (Clarke et al. 1993), although this effect is non-specific. Unfortunately I was unable to release my cells from G2 arrest using okadaic acid. In addition, okadaic acid in REF52 cells may be toxic, possibly through induction of either apoptosis or mitotic catastrophe (unpublished results). Thus my results are uninformative as to whether PP2A has a role in p53 induced G2 arrest, although the possibility remains open.

Is Wee1 involved?

I should stress that although my results are suggestive of p53 acting through Cdc25C

to control G2 progression, there is no reason why it may not also act through the other major regulator, Wee1 kinase. As mentioned in the introduction, Wee1 is regulated by some of the same kinases and phosphatases as Cdc25C, which alters its cellular location and activity. In fission yeast, Wee1 is phosphorylated and activated by Chk1, which results in maintenance of Tyr 15 phosphorylation and G2 delay (O'Connell et al. 1997). The results from my Cdc25C experiments hint that Chk1 kinase may be active during p53 induced G2 arrest. This might indicate that as well as downregulating Cdc25C activity in my cells, Chk1 could be upregulating Wee1 activity to further promote phosphorylation of Tyrosine 15. PP2A maintains Wee1 in an active, unphosphorylated state (Clarke et al. 1993; Kinoshita et al. 1993), thus p53 activation of PP2A could upregulate Wee1 activity while downregulating Cdc25C. Alternatively, p53 might affect the expression/function of other Wee1 control proteins such as Nim1, or its regulator Nif1. 14-3-3σ has been reported to bind to Wee1, which may also affect Wee1 localization and function by sequestering it in a manner similar to Cdc25C (Honda et al. 1997; O'Connell et al. 1997). The status of Wee1 in p53 induced G2 arrest needs to be determined before any of these possibilities can be addressed.

What does Cyclin B1 location/status tell about the G2 arrested cells?

Another mechanism in cell cycle control which is of equal importance for proper cell division is the physical location of the cell cycle proteins. Nuclear translocation mediates the biological activity of Cyclin B1. Results from a number of systems indicate that Cyclin B1 levels accumulated substantially before mitosis, peaking at metaphase, and decline rapidly as the cells proceed through anaphase (Pines and Hunter, 1991; Kakino et al. 1996;

Widrow et al. 1997). Cyclin B1 is confined to the cytoplasm until the end of G2, surrounding the nucleus in an intense halo beginning in late S phase/early G2 (Pines and Hunter, 1991; Widrow et al. 1997). The protein only enters the nucleus to initiate mitosis, after the cells have begun prophase, and before nuclear lamina breakdown (ibid). Cyclin B1 is completely lost by telophase, the majority being destroyed at the metaphase-anaphase transition (ibid). Nuclear translocation of Cyclin B1 may require phosphorylation within the CRS (cytoplasmic retention signal) domain (Li et al. 1997). The CRS appears to be responsible for retention although the mechanism remains unknown. When all serine residues are mutated to the Ala mutant, cyclin is inactivated, and the CRS mutant can be reactivated by adding a nuclear location signal (NLS) or second CRS domain with Ser mutated to Glu (mimics phosphorylation) (Li et al. 1997). In contrast, Cyclin A is predominantly nuclear and does not appear to be associated or retained in the cytoplasm, suggesting different mechanisms of control (Pines and Hunter, 1991). Increase in cytoplasmic staining of Cyclin B1 is seen after irradiation of SCC61 human squamous cell carcinoma cells, and decrease in nuclear staining associated with G2 arrest. Cdc2 kinase was also hyperphosphorylated and presumably inactive (Smeets et al. 1994).

The staining for Cyclin B1 location in the p53 blocked cells resembles the interphase staining seen by Pines and Hunter (91) in HeLa cells. The protein is for the most part concentrated around the nucleus, although I cannot exclude the possibility that a small proportion has translocated into the nucleus. This suggests that the cells have arrested at some point in G2 prior to mitosis.

Is p53 altering the timing of translocation events? In its role as a transcription factor

it could be affecting the expression of proteins involved in Cyclin B1 translocation, such as the kinases (yet unidentified) which work on the CRS region. Nuclear translocation of Cyclin B1 is not dependent on microtubules or actin filaments (Smeets et al. 1994; Ookata et al. 1992). Cyclin B1 is attached to detergent-resistant cytoskeleton, which is associated with the mitotic apparatus/microtubules/spindle apparatus (Ookata et al. 1992; Smeets et al. 1994). P53 has been shown to control centrosome duplication and is implicated in the mitotic spindle checkpoint (Frattini et al. 1997; Cross et al. 1995; Fukasawa et al. 1996). Some of the same mechanisms important for induction of the mitotic spindle checkpoint (such as Plk1) might also be employed to promote arrest at G2 by preventing translocation of the Cdc2/Cyclin B1 complex. Little is known of the spindle status of my arrested cells. The results from one tubulin staining showed very disorganized, tangled formations which did not resemble any known structures. These cells also had variable numbers of centrosomes, ranging from one to multiple copies (unpublished data). These experiments need to be repeated to better characterize these structures. It also may be useful to track Cyclin B1 location over a long term arrest. Does it remain around the nucleus, disassociate throughout the cytoplasm or become degraded? I do observe a modest decrease in Cyclin B1 expression later (12 hours onwards) when the cells remain in G2 arrest.

What about the localization of other cell cycle proteins?

It would also be useful to determine the location of Cdc2 kinase. Regulation of the subcellular localization of Cdc2 kinase is associated with Cyclin B1 (Booher et al. 1989; Pines and Hunter, 1991; Smeets et al. 1994). The presumption is that Cdc2 is associated

with Cyclin B1 since the results from the coimmunoprecipitation experiments suggest that the two proteins are complexed during the initial arrest, although there may be some disassociation later into arrest. I observed a slight decrease in Cyclin B1 expression as the cells remain at the G2 arrest point, which might explain in part why the steady state levels of this complex drops.

Control over translocation might also be important in preventing Cdc25C interaction with the Cdc2/Cyclin B1 complex. Cdc25C is a cytoplasmic protein which in HeLa and BHK cells enters the nucleus at the G2/M transition, similar to Cyclin B1 translocation (Heald et al. 1993; Seki et al. 1992). Nuclear translocation is required to initiate nuclear mitotic events (Heald et al. 1993). The recent report of p53 stimulation of 14-3-3σ expression may provide one potential mechanism for preventing Cdc25C from interacting with Cdc2/Cyclin B1 (Hermeking et al. 1997). Binding of 14-3-3σ does not affect Cdc25C activity (Peng et al. 1997). Instead, it has been proposed that 14-3-3σ may sequester Cdc25C away from other cell cycle proteins in the cytoplasm, and prevent its transit to the nucleus (Peng et al. 1997). No evidence has yet been provided as to whether this is how 14-3-3σ functions during p53 induced arrest. The cellular localization of Cdc25C and 14-3-3σ needs to be determined during p53 G2 arrest to address this possibility.

All of this does not necessarily diminish the importance of tyrosine phosphorylation in controlling Cdc2 activity. There is evidence that Cdc2 kinase may be first activated in the cytoplasm before translocation to the nucleus. During *Xenopus* oocyte maturation, Cyclin B1/Cdc2 complexes are activated in the cytoplasm prior to nuclear translocation (Ookata et al. 1992), moving into germinal vesicles before nuclear envelope breakdown. This implies

that dephosphorylation and activation before translocation may be important events in cell cycle progression. Wee1, which is primarily a nuclear protein, ensures completion of DNA replication prior to mitosis. This may involve protecting the nucleus from cytoplasmicly active Cdc2 (Heald et al. 1993). Together, this may point to a potential cytoplasmic function for Cdc2. Cdc2 is maintained in an inactive state in the p53 G2 arrested cells. Perhaps as well as keeping active Cdc2/Cyclin B1 from entering the nucleus and initiating mitosis, p53 is preventing the kinase from performing other tasks normally performed in the cytoplasm.

The cellular location of Cdc2 kinase is also implicated in G2 delay in response to radiotherapy (Cohen-Jonathan et al. 1997). These authors noted the preferential localization of Cdc2 to the cytoplasm after irradiation in primary tumours from patients with a history of recurrent tumours. No significant difference was seen in either Cyclin A or B1 levels (ibid). Thus, phosphorylation of Cdc2 may be important for mediating the G2/M transition for two reasons. One, to prevent activation of the kinase, and two, to prevent transit of the Cdc2/Cyclin B1 complex to the nucleus.

The potential model for p53 action at G2.

I have discussed a number of possibilities for p53 action at G2. These can be tied together in a general model diagrammed in Figure 1. Wild type p53 responds to stimulus at G2 by initially inducing or maintaining phosphorylation of Cdc2 kinase on Tyr 15, and potentially Thr14. This may occur either by p53 influencing the action of the proteins controlling Cdc25C function (potentially of Chk1, Plk1 or PP2A), and by the upregulation of 14-3-3σ gene expression, and/or by direct or indirect upregulation of Wee1. This action would have at least two consequences. First, it would prevent activation of the Cdc2/Cyclin

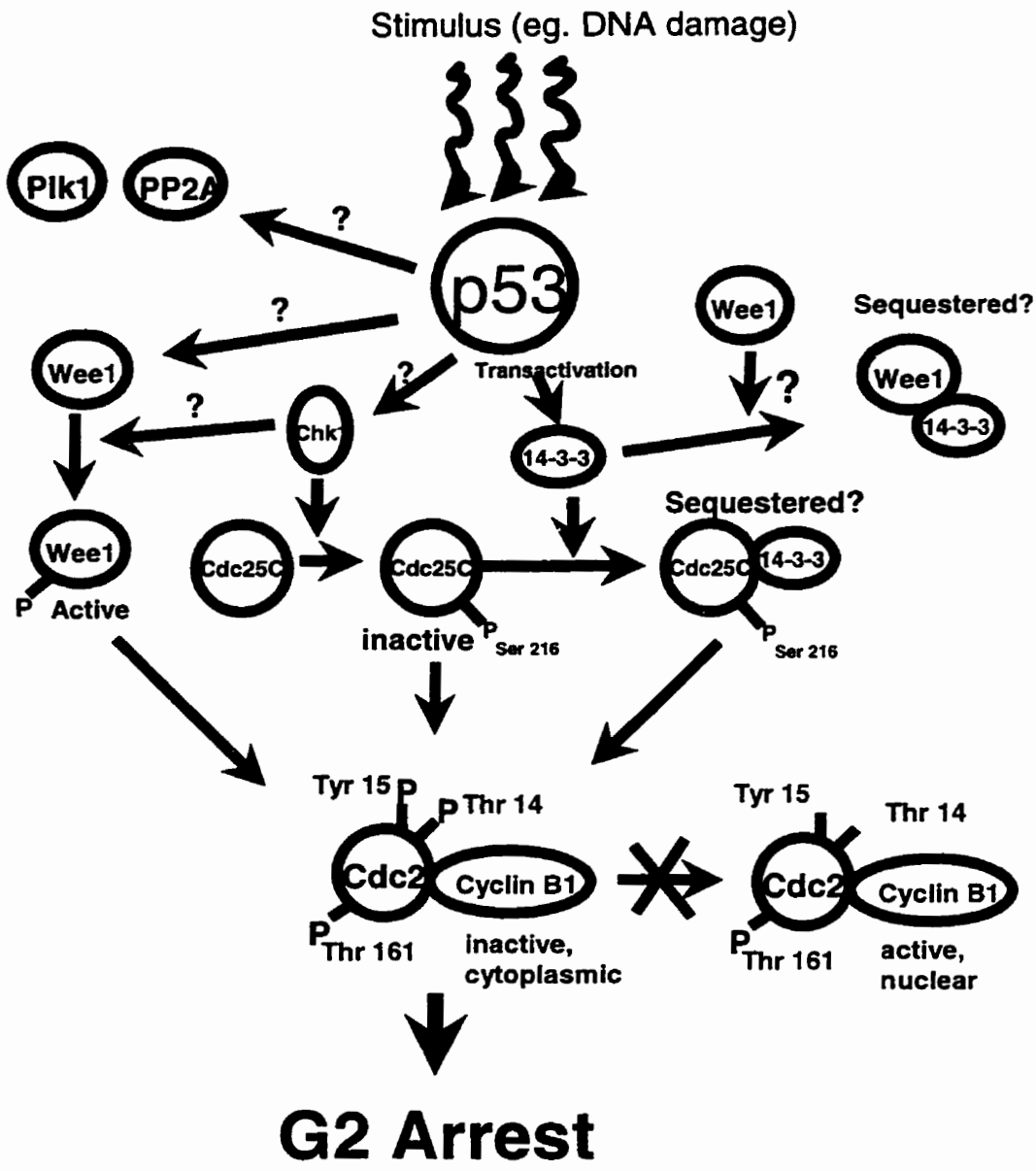


Figure 1: Proposed model for p53 action during G2 arrest. Diagramatic representation of the potential pathways used by p53 to induce arrest at G2, as outlined in text. Adapted in part from Hermeking et al., 1997, and Nurse, 1997.

B1 complex. Second, it could prevent translocation of the Cdc2/Cyclin B1 complex to the nucleus. Both events would prevent the G2/M transition and progression through mitosis. P53 action on the Plk1 kinase or other yet unidentified factors may also serve to prevent progression into mitosis by preventing proper centrosome and spindle apparatus formation.

Conclusion.

There remain a great number of possibilities by which p53 may act during G2 arrest. While control over Cdc2 kinase function appears essential, the components with which p53 interacts to induce this arrest remain to be discovered. The determination of these players will provide for interesting times in the future.

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IMAGE EVALUATION
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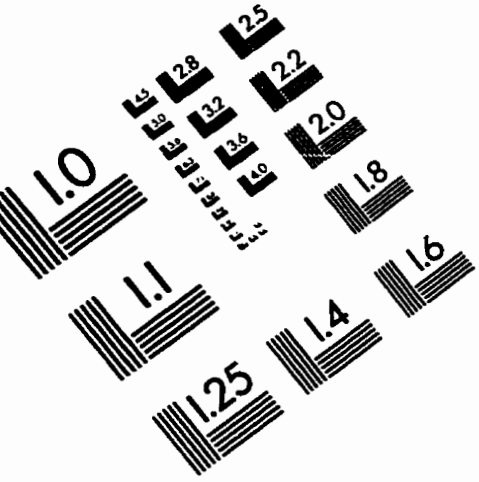

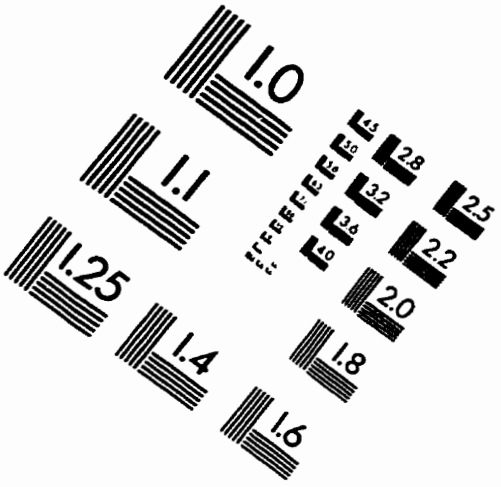

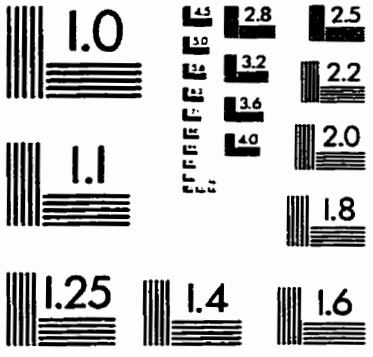

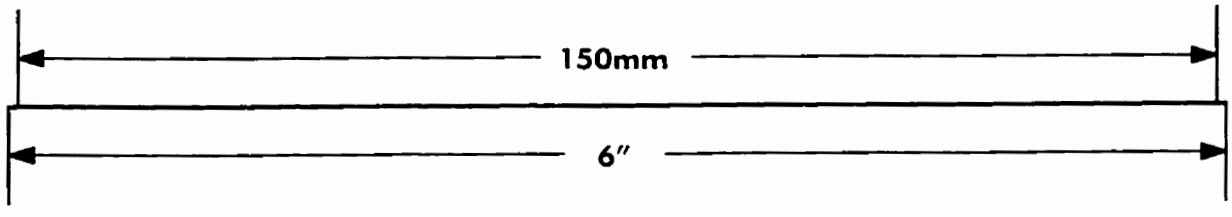

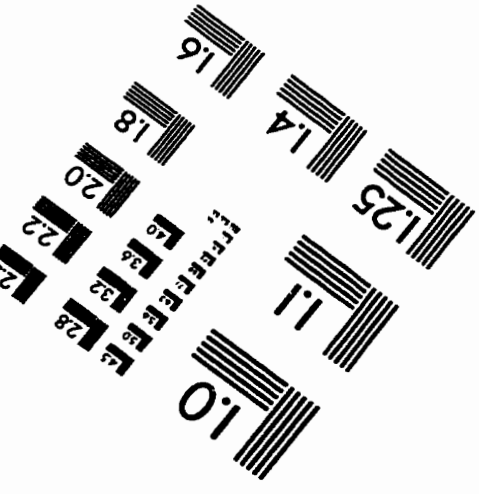

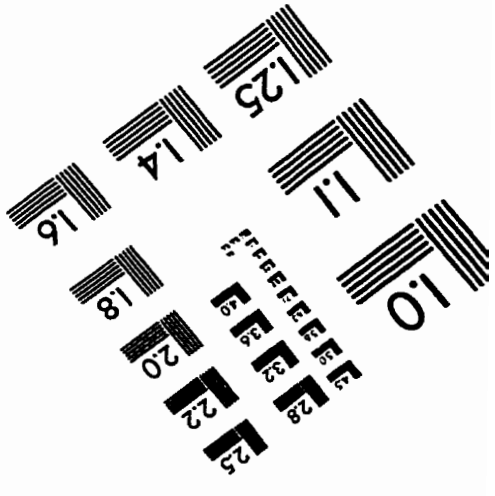

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