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Cyclin G is a transcriptional target of the p53 tumor suppressor protein

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Through a PCR-based differential screening method, cyclin G was identified as a novel transcriptional target of the p53 tumor suppressor gene product. In both a mouse p53 temperature-sensitive leukemic cell line and mouse embryonic fibroblasts (MEF) after γ-irradiation, cyclin G mRNA was rapidly induced. MEF from a p53-deficient mouse expressed cyclin G at a level >10-fold lower than that from a wild-type mouse. Using a DNA binding assay, a specific p53 binding site was identified upstream from the cyclin G gene, which functioned as a p53-dependent *cis*-acting element in a transient transfection assay. These results suggest that cyclin G might participate in a p53-mediated pathway to prevent tumorigenesis.

Key words: cyclin/differential screening/gene expression/p53

Introduction

p53 is regarded as one of the most important tumor suppressors, whose alterations are among the most common genetic changes found in human tumors (Levine *et al.*, 1991). p53 protein is induced by DNA damage (Nelson and Kastan, 1994) and the induced expression of p53 causes either cell cycle arrest or apoptosis, depending on cell type. Hence the emerging picture arises that p53 functions as a 'guardian of the genome' (Hartwell, 1992; Lane, 1992). It has been hypothesized that p53 may cause cell cycle arrest or apoptosis to prevent the accumulation of genetic damage which can eventually contribute to neoplastic transformation.

p53 has been shown to function as a transcription factor (Donehower and Bradley, 1993), and to date, four transcriptional targets of p53 have been identified. These target genes are *gadd45* (Kastan *et al.*, 1992), *mdm2* (a negative regulator of the *trans*-activating function of p53, Wu *et al.*, 1993; Juven *et al.*, 1993), *mck* (Zambetti *et al.*, 1992) and p21/*WAF* (an inhibitor of cyclin-dependent kinases, El-Deiry *et al.*, 1993; Gu *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993). The transactivation of *mdm2* and p21/*WAF* by p53 helps to explain how the activity of p53 is regulated and how the induction of p53 causes cell cycle arrest, respectively. The functions ascribed to the downstream targets of p53 identified thus far cannot, however, account completely for phenotypes associated with p53 function. In fact, it is not currently understood how p53 causes apoptosis. Therefore, there may be other transcriptional targets of p53. The identification of additional p53-regulated genes is critical to gain a full understanding of p53 function.

In order to identify novel target genes of p53, we applied a differential screening approach. Here we report the identification of the mouse cyclin G gene as a transcriptional target of p53. We will discuss the potential role of cyclin G in the p53-mediated pathway.

Results

Identification of the cyclin G gene as a potential transcriptional target of p53 through a differential screening method

In order to identify novel transcriptional targets of p53, we have used a PCR-based differential screening method described before (Liang and Pardee, 1992; Liang *et al.*, 1993). LTR6, a mouse leukemic cell line transformed by a p53 temperature-sensitive mutant ($p53^{val135}$), and M1 (S6 subclone), the parental cell line of LTR6 (S6 cells fail to express detectable p53), were used for this screen (Yonish-Rouach *et al.*, 1991, 1993). At 39°C, $p53^{val135}$ takes on an inactive mutant conformation and no difference in phenotype is observed between LTR6 and S6 cells. However, at 32°C, $p53^{val135}$ takes on an active wild-type conformation and LTR6 cells lose their viability over a 48 h period after temperature shift, whereas S6 cells continue to grow exponentially as at 39°C (Yonish-Rouach *et al.*, 1991, 1993). RNA was harvested from LTR6 at the restrictive temperature (39°C) and 2 h after a temperature shift to the permissive temperature (32°C). RNA was also harvested from S6 under similar conditions. The fingerprint patterns of PCR products derived from RNA harvested under these two conditions were compared, and the bands which appeared differentially in LTR6 but not in S6 were picked up as potential transcriptional target genes of p53 (Figure 1A, see also Materials and methods).

For this screen, we used 16 arbitrary 10-mers in total and identified 13 positive bands (all of them showed increased levels of expression after temperature shift in LTR6). After re-amplification and subcloning, three of 13 clones showed the expected patterns of expression on Northern blots. Sequence analysis revealed that one of these positive clones (derived from the band shown in Figure 1A) showed 85% homology over 320 bp to the 3′ untranslated region of the rat cyclin G cDNA (Tamura *et al.*, 1993; Figure 2). Northern blot analysis of this clone is shown in Figure 1B. The expression of this clone (transcript corresponding to 3.5 kb) was induced on temperature shift in LTR6 but not in S6, whereas the expression of cyclin B1 did not change after temperature shift in either cell line, suggesting that it was expressed in a p53-dependent manner. Using this clone as a probe,

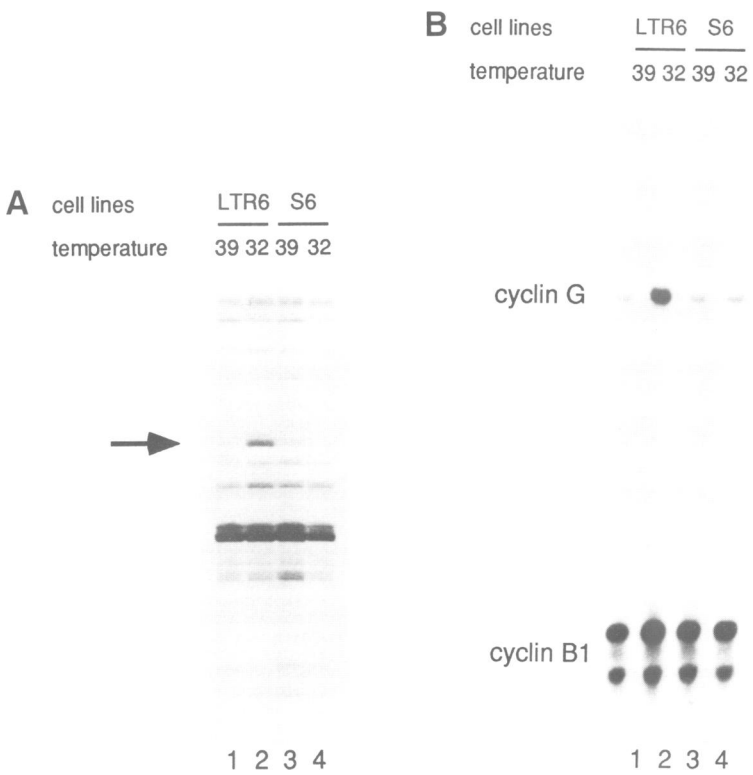

Fig. 1. PCR-based differential screen. Each total RNA was treated with RNase-free DNase I, reverse-transcribed using an anchored oligo(dT) primer, and amplified by PCR using an arbitrary 10-mer and an anchored oligo(dT) primer (Liang and Pardee, 1992; Liang *et al.*, 1993; also see Materials and methods). Amplified cDNAs were run on 6% sequencing gels side by side, and the bands which were differentially expressed in LTR6 but not in S6 (a subclone of M1) were identified as positive bands. cDNAs were recovered from the positive bands and re-amplified using the same set of primers used in the initial amplification. Re-amplified cDNAs were gel-purified on an agarose gel, subcloned into a plasmid and sequenced. cDNA inserts were used as probes for Northern blot analysis. (**A**) Differential display of amplified cDNA on a 6% sequencing gel. Lane 1, LTR6 at 39°C; lane 2, LTR6 2 h after temperature shift to 32°C; lane 3, S6 at 39°C and lane 4, S6 2 h after temperature shift to 32°C. The positive band is marked by an arrow (lane 2, 0.32 kb). (**B**) Northern blot analysis. The positive clone obtained by screening (cyclin G), and cyclin B1 (Chapman and Wolgemuth, 1992) were used as probes. Lane 1, LTR6 at 39°C; lane 2, LTR6 2 h after temperature shift to 32°C; lane 3, S6 at 39°C and lane 4, S6 2 h after temperature shift to 32°C.

a mouse embryonic stem cell cDNA library was screened for a full length cDNA. All of six positive clones analyzed showed the same restriction enzyme digest patterns suggesting that they were derived from the same gene. Sequence analysis of the longest clone showed that it encodes a mouse homolog of cyclin G (Figure 2). Mouse cyclin G cDNA contains an open reading frame of 249 amino acids with 96% identity to rat cyclin G.

Specific induction of cyclin G expression by p53

In order to confirm that cyclin G is regulated by p53, we examined the expression of cyclin G as well as p21 and cyclin B1 by Northern blot analysis in cells with altered levels of p53. First, their expression was analyzed over a time course after temperature shift in LTR6 and S6 (Figure 3A). In LTR6, the expression of cyclin G was induced in 1 h (6-fold induction compared with basal level expression) and increased until it reached its peak at 8 h with 30-fold induction, whereas in S6, it did not increase more than 2-fold throughout the time course. The expression of p21 in LTR6 was undetectable before the shift and increased with similar kinetics to cyclin G induction. The expression of p21 in S6 was undetectable throughout the time course. The level of cyclin B1 mRNA did not increase more than 2-fold in both LTR6 and S6. The expression of all three genes slowly began to decrease after 12 h in LTR6, which probably resulted from apoptotic cell death caused by p53 (Yonish-Rouach *et al.*, 1991, 1993). Previous results of Northern blotting analysis demonstrated that the levels of cyclin A, D1 and D2 mRNA remain unchanged in LTR6 for at least 5 h after a temperature shift to the permissive temperature (Levy *et al.*, 1993), indicating that the increase in cyclin G expression in LTR6 is unique among cyclins.

Second, similar time course studies were done in MEF from a wild-type mouse after γ-irradiation (Figure 3B). γ-irradiation induces p53-dependent G_1 cell cycle arrest in MEF (Lowe *et al.*, 1993). Again, the cyclin G mRNA was induced in 1 h (3-fold) and increased 15-fold in 12 h. The level of p21 mRNA did not increase at all throughout the time course. The expression of the major cyclin B1 mRNAs (2.5 and 1.7 kb) did not change throughout the time course, although two minor transcripts (4.0 and 2.0 kb) increased with slow kinetics. We have also done time course studies after γ-irradiation using MEF from a p53-deficient mouse. However, we did not observe a significant increase in the level of cyclin G transcript on Northern blot over a period of 24 h (unpublished data).

Finally, we compared the basal expression level of three genes in non-irradiated MEF from a p53-deficient mouse and from a wild-type mouse (Figure 3C). The expression of p21 and cyclin G was much lower (>10-fold) in MEF from a p53-deficient mouse compared with MEF from a

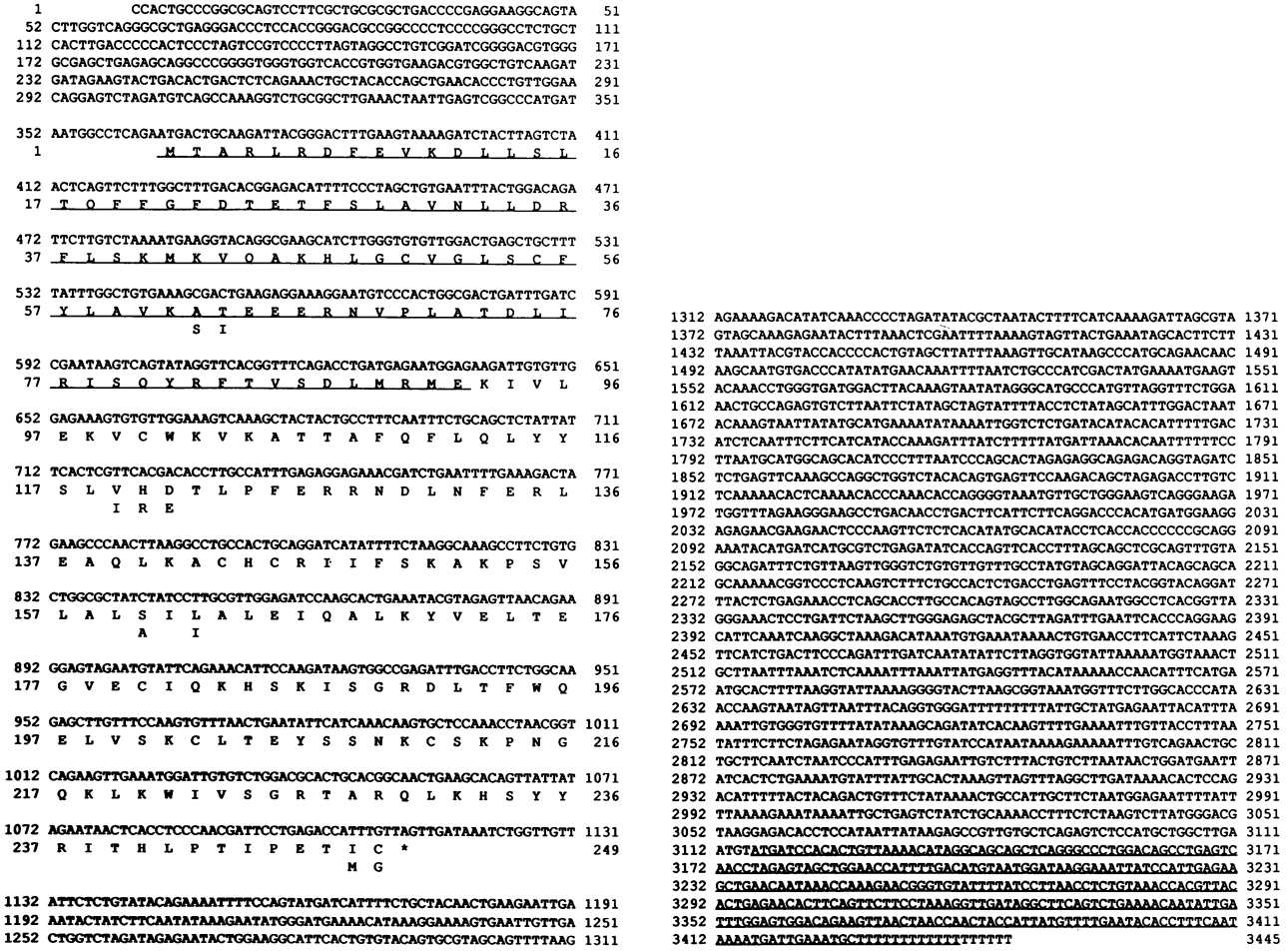

Fig. 2. Sequence of mouse cyclin G cDNA. The underlined sequence at the 3′-terminal 324 bp is the fragment originally identified during the differential screening. The predicted amino acid translation of mouse cyclin G is shown below the nucleotide sequence. The underline below the N-terminal 92 amino acids shows the location of the cyclin box. The rat cyclin G amino acid sequence is shown under the mouse cyclin G sequence (only residues which differ from the mouse cyclin G sequence are shown).

wild-type mouse. In contrast, the expression of cyclin B1 was ~5-fold higher in MEF from a p53-deficient mouse compared with MEF from a wild-type mouse. These data from Northern blot analysis are consistent with the hypothesis that cyclin G expression is induced by p53. The rapid kinetics of induction in LTR6 and MEF suggest that cyclin G is a direct transcriptional target of p53.

Identification of a specific p53 binding site in genomic cyclin G gene sequence

To understand the mechanism of induction of cyclin G mRNA expression by p53, we analyzed a genomic clone of the cyclin G gene. Assuming that cyclin G is a direct transcriptional target of p53, we searched for genomic fragments bound by p53 by an immunoprecipitation assay (El-Deiry *et al.*, 1992). A phage DNA containing the genomic cyclin G gene was digested with *Hin*fI, end-labeled and mixed with lysates prepared from Sf9 cells expressing p53. The immunoprecipitates with anti-p53 antibody PAb421 were separated on a polyacrylamide gel (Figure 4A, left panel). A 0.34 kb fragment (HH0.34, marked by the arrow in Figure 4A, left panel) was immunoprecipitated only when both p53 and the antibody against p53 were present, suggesting that this fragment was directly bound to p53. This p53 binding fragment was cloned into pBluescript plasmid and the insert DNA was again used in the immunoprecipitation assay (Figure 4A, lane 6). As negative controls, the other *Hin*fI-digested genomic cyclin G fragments (fr.A and fr.B), which were similar in length to the p53 binding fragment, were also used in this assay (Figure 4A, lanes 8 and 10). Approximately 1% of the p53 binding fragment was immunoprecipitated (Figure 4A, lanes 5 and 6) whereas <0.05% of control fragments were immunoprecipitated (Figure 4A, lanes 7 and 8, lanes 9 and 10), suggesting that the p53 binding fragment contains a specific p53 binding site. Sequence analysis demonstrated that the p53 binding fragment contained a sequence similar to the p53 binding consensus sequence (El-Deiry *et al.*, 1992). This sequence differs from the p53 consensus at only one position (Figure 4B). It is located 1.5 kb upstream of the initiation methionine of cyclin G in the genomic DNA (unpublished data).

To confirm that p53 is capable of binding specifically to this p53 consensus-like sequence, a 34 bp oligonucleotide containing this sequence (designated WT30, Figure 4B) was used in a gel shift assay (Figure 4C). The retarded bands that appeared in the presence of lysates prepared from Sf9 cells expressing p53 were supershifted if PAb421 was added (two upper arrows in Figure 4C), indicating

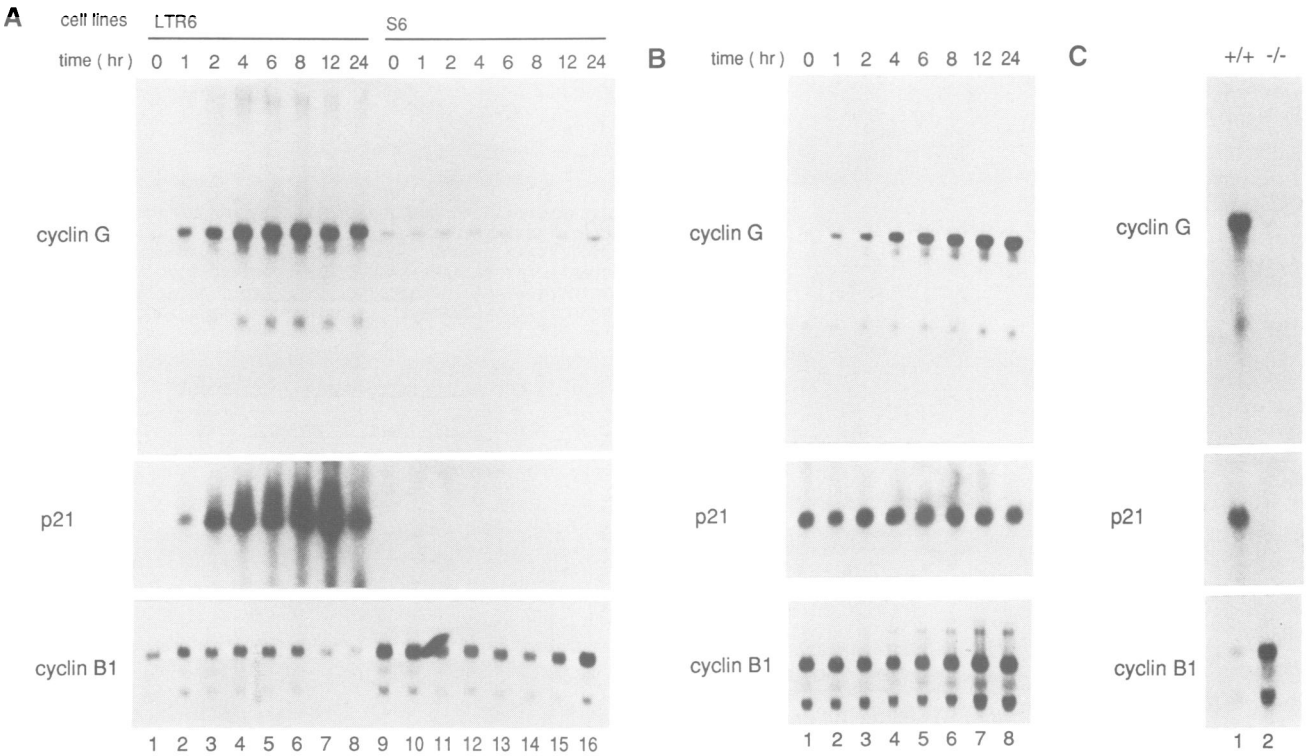

Fig. 3. Northern blot analysis of cyclin G, p21 and cyclin B1. 10 μg of total RNA were loaded per lane. (**A**) Time course experiment after temperature shift (from 39°C to 32°C) in LTR6 and S6. (**B**) Time course experiment after γ-irradiation (5 Gy) in MEF (passage 2). (**C**) Comparison between wild-type MEF (+/+) and p53-deficient MEF (−/−).

that WT30 was bound by p53. If WT30 or a 20 bp oligonucleotide corresponding to a perfect p53 consensus sequence was used as competitor at a 50-fold excess molar ratio, the supershifted bands were almost completely abolished. In contrast, if MUT30, a 30 bp oligonucleotide in which WT30 is changed at three positions so that the internal symmetrical structure of WT30 is destroyed (Figure 4B), was used as a competitor, the supershifted bands were not abolished at all. These results show that p53 binds specifically to WT30.

Transactivation of the specific p53 binding site from genomic cyclin G gene sequence by p53

Finally, we wished to determine if WT30 can function as a p53-dependent *cis*-acting element *in vivo*. For this purpose, we performed a transient co-transfection assay in p53-deficient MEF. Either WT30, MUT30 or HH0.34 was inserted upstream of the herpes simplex virus tymidine kinase promoter driving a luciferase gene (ptk-luc), generating pWT30-tk-luc, pMUT-tk-luc and pHH0.34-tk-luc, respectively. These plasmids were co-transfected either with a CMV-driven p53 expression vector (Baker *et al.*, 1990) or with a CMV vector with no insert, and luciferase activity was assayed 48 h after transfection. The luciferase activity was then normalized to β-galactosidase activity derived from the co-transfected β-galactosidase construct. The averages of two independent experiments are shown in Figure 4D. With ptk-luc, luciferase activity was increased no more than 2-fold upon p53 expression. Likewise, pMUT30-tk-luc failed to show a significant increase in luciferase activity upon p53 induction (1.4-fold). In contrast, pWT30-tk-luc and pHH0.34-tk-luc showed 58- and 13-fold increases in luciferase activity upon p53 expression respectively. These results demonstrate that WT30 can function as a p53-dependent *cis*-acting element *in vivo*. The results of the DNA binding and transfection assays indicate that the cyclin G gene contains a functionally active p53 binding site. Taken together with the results from Northern blot analysis, we conclude that cyclin G is a novel transcriptional target gene of p53.

Discussion

Cyclin G was originally identified by spurious cross-hybridization of its cDNA with a mixture of c-*src* family proto-oncogene probes (Tamura *et al.*, 1993). Cyclin G has no destruction box, and among cyclins most resembles fission yeast Cig1 cyclin, a B-type cyclin which might function early in the cell cycle (Bueno *et al.*, 1991; Connolly and Beach, 1994), and human cyclin A (Tamura *et al.*, 1993). So far, neither the physiological role nor the biochemical function of cyclin G has been defined.

From the results of the experiments shown here, we conclude that cyclin G is a direct transcriptional target of p53. First, the expression of cyclin G is induced under several different conditions which are known to induce p53 expression (Figure 3), and the induction of cyclin G expression is rapid upon p53 induction (Figure 3A and B). Second, a specific p53 binding site was identified upstream of the cyclin G gene using a DNA binding assay (Figure 4A, B and C). Finally, this p53 binding site functions as a p53-dependent *cis*-acting element in a transient transfection assay (Figure 4D).

There are several possible roles of cyclin G in the p53-mediated pathway. First, cyclin G might function to cause

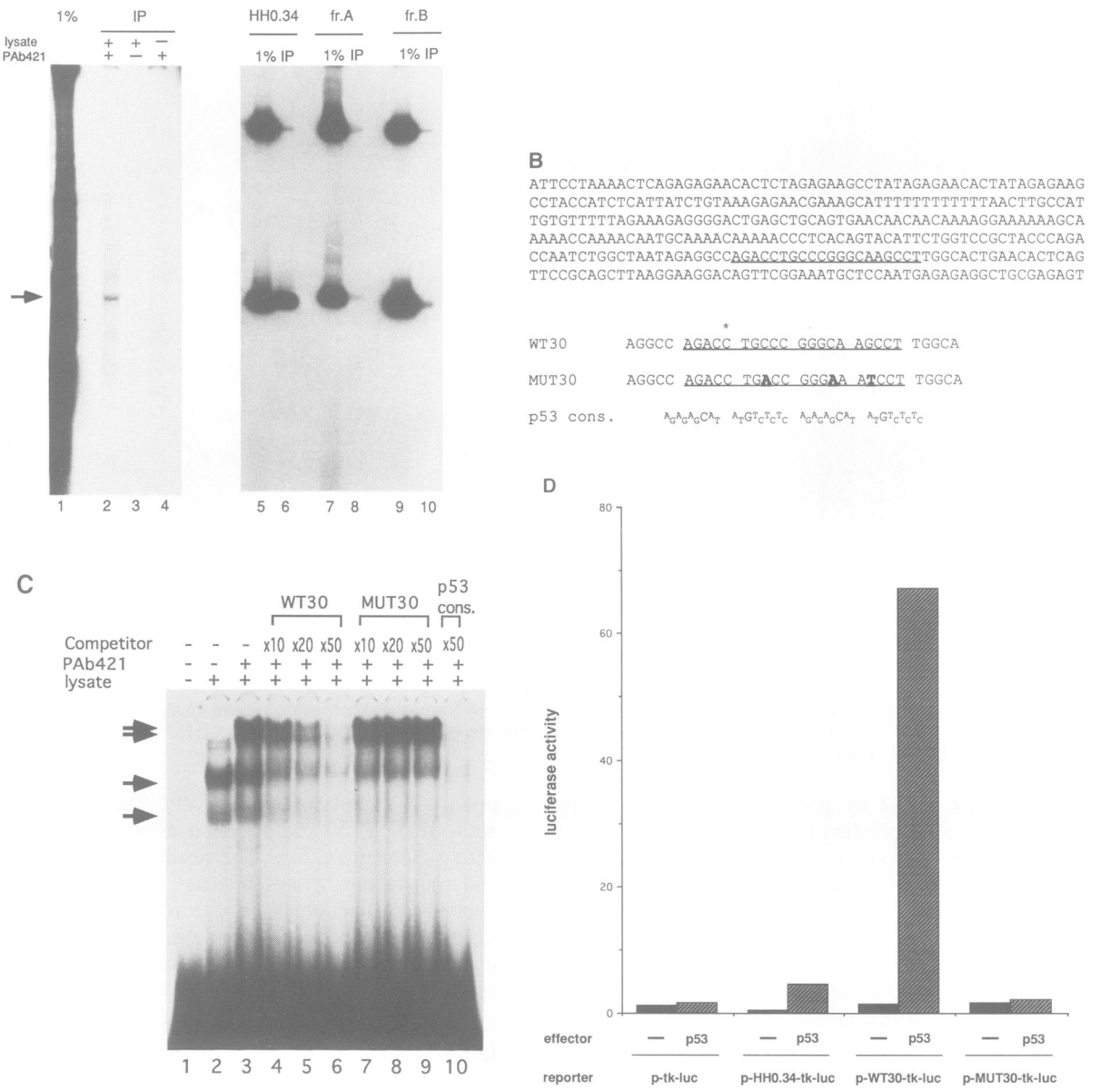

Fig. 4. (**A**) Left panel: identification of a genomic cyclin G DNA fragment that bound to p53 by an immunoprecipitation assay. λEMBL3A phage DNA containing cyclin G gene was digested with *Hin*fI, end-labeled and used for the assay. Lane 1, 1% of end-labeled input DNA. Lane 2, labeled DNA was incubated with lysates of baculovirus-infected Sf9 cells synthesizing wild-type p53. After immunoprecipitation with anti-p53 antibody PAb421, a bound DNA fragment was recovered and separated on a non-denaturing polyacrylamide gel. Lane 3, same as lane 2 except that PAb421 was not included during immunoprecipitation. Lane 4, same as lane 2 except that lysates from uninfected Sf9 cells were used instead of lysates of baculovirus-infected Sf9 cells synthesizing wild-type p53. The positive band (HH0.34) is indicated by an arrow. Right panel: specific binding of the isolated p53 binding fragment (HH0.34) to p53. HH0.34, fragment A and fragment B (fragments A and B are the other genomic cyclin G fragments that have similar length to HH0.34) were subcloned into the plasmid pBluescript, and the inserts were separated from the vector by restriction enzyme digestion. The digested plasmids were end-labeled and used for an immunoprecipitation assay. Lanes 5, 7 and 9 contain 1% of the total end-labeled DNA used for an immunoprecipitation assay. In lanes 6, 8 and 10, end-labeled DNA was immunoprecipitated with p53. The digested plasmid containing HH0.34 (lanes 5 and 6), fragment A (lanes 7 and 8) and fragment B (lanes 9 and 10) were used as end-labeled DNA. The upper and lower bands represent the positions of the plasmid vector and the genomic fragments, respectively. (**B**) Top panel: sequence of HH0.34. The underlined sequence matches the p53 consensus sequence with 1 bp disparity. Bottom panel: sequence of oligonucleotides used for the gel shift assay in (C). WT30 corresponds to the p53 consensus-like sequence in HH0.34 (the nucleotide that is different from p53 consensus is marked with an asterisk). MUT30 is identical to WT30 except that three nucleotides are changed (changed nucleotides are shown as bold characters). The p53 consensus sequence described before (El-Deiry, 1992) is shown for comparison. (**C**) Specific binding of p53 to WT30 probe in a gel shift assay. End-labeled WT30 probe was incubated in the presence (+) or absence (−) of lysates of baculovirus-infected Sf9 cells synthesizing wild-type p53, in the presence (+) or absence (−) of PAb421 and in the presence of a 10-fold excess (×10), 20-fold excess (×20), 50-fold excess (×50) or absence (−) of competitors shown in the figure. DNA−protein complexes were separated on a non-denaturing polyacrylamide gel. The p53 consensus sequence used here as a competitor is AGGCATGCCTAGGCATGCCT. (**D**) Transactivation of pWT30-tk-luc by p53 in a transient transfection assay. p53-deficient MEF were transfected by a calcium phosphate transfection method. WT30, MUT30 or HH0.34 were cloned 5′ to the herpes simplex virus tymidine kinase promoter element of the luciferase reporter plasmid ptk-luc, to generate pWT30-tk-luc, pMUT-tk-luc and pHH0.34-tk-luc, respectively. Plasmids were co-transfected with the CMV-driven p53 expression vector pC53SN3 (Baker *et al.*, 1990) or the CMV vector with no insert. The p53 expression vector and CMV vector are shown as p53 and −, respectively. After glycerol shock, cells were harvested 48 h post-transfection and luciferase activities were determined by luminometer. Luciferase activities were normalized to the activity of β-galactosidase. The averages of two independent experiments are shown.

cell cycle arrest. It is well-known that, in many cell lines, the induction of p53 expression causes cell cycle arrest (Diller *et al.*, 1990; Kastan *et al.*, 1991; Martinez *et al.*, 1991). The fact that cyclin G expression is induced in MEF in the absence of p21 induction (Figure 3B) supports this possibility. In this regard, it is tempting to imagine that cyclin G may function as an inhibitory subunit of cdk(s). It is possible that high level expression of cyclin G inhibits one (or some) of the G_1 cyclin(s) by competing for its (their) catalytic cdk partner(s), thereby functioning as an anti-cyclin and providing cells with another means to inhibit cyclin−cdk kinase activity in addition to that provided by p21. In order to determine if overexpression of cyclin G causes cell cycle arrest, we constructed a CMV-driven cyclin G expression vector and co-transfected it with a CD20 expression vector into either SAOS2 (human osteosarcoma cell line) or NIH3T3 (mouse fibroblast cell line), followed by FACS analysis (Zhu *et al.*, 1993). (We have not performed this assay using MEF due to low transfection efficiency.) The experiments showed, however, that the overexpression of cyclin G did not cause cell cycle arrest in these cell lines (unpublished data). This result argues against this hypothesis, although it is still possible that cyclin G is involved in cell cycle arrest in cell types other than SAOS2 and NIH3T3.

Second, cyclin G might function to cause apoptosis, another well-known phenotype caused by the induction of p53. This hypothesis is supported by the fact that cyclin G is induced after p53 induction in LTR6 (Figure 3A), a cell line in which the induction of p53 causes apoptosis. Recently it was shown that cdc2 kinase is involved in apoptosis mediated by a lymphocyte granule protease (Shi *et al.*, 1994). It is an attractive possibility that cyclin−cdk kinase complexes also play important roles in other types of apoptosis: a cyclin G−cdk complex might be responsible for p53-mediated apoptosis. Since both cyclin G and p21 are induced in LTR6 by p53, any cyclin G−cdk complex formed may be resistant to inhibition by p21 if it forms an active complex.

Third, it may be possible that cyclin G is involved in DNA repair. Recently, it has been shown that DNA strand breaks are sufficient and probably necessary to elevate p53 protein levels (Nelson and Kastan, 1994). It would be reasonable, therefore, to assume that p53 directs the expression of genes that are involved broadly in the DNA repair process. It is possible that the cyclin G−cdk complex phosphorylates and activates protein(s) involved in DNA excision repair, thereby providing a link between p53 and DNA repair processes. This possibility is supported by the observations that many proteins are involved both in DNA replication and in DNA repair (Prelich *et al.*, 1987; Coverley *et al.*, 1991, 1992; Shivji *et al.*, 1992) and cyclin−cdk complexes may play an important role in initiation of DNA replication (Dutta and Stillman, 1992; Fotedar and Roberts, 1992).

It was surprising that there is no induction of p21 after γ-irradiation in MEF. The expression of p21 was induced 10-fold in 2 h after exposure to a similar dose of γ-irradiation (4 Gy) in ML1, a human leukemic cell line containing wild-type p53 (G.Hannon, unpublished data); the response of p21 to γ-irradiation may be cell type specific. Alternatively, it may be dose dependent and a higher dose of γ-irradiation may be needed to induce p21 expression in MEF.

We have identified cyclin G as a novel transcriptional target of p53. We believe that this observation will aid in elucidating one of the pathways of p53, whose inactivation plays a crucial role in the development of human cancers.

Materials and methods

Cell culture

LTR6 and S6 cells were cultured in RPMI1640 with 10% fetal calf serum. MEF cells were cultured in Dulbecco's MEM with 10% fetal calf serum.

PCR-based differential screening

PCR-based differential screening was performed essentially as previously described (Liang and Pardee, 1992; Liang *et al.*, 1993). Total RNAs were extracted from cells with RNAzolB (TEL-TEST, INC) and chloroform, and precipitated with isopropanol. After suspension in DEPC-treated dH_2O, 20 μg of each RNA was treated with 20 U of RQ RNase-free DNase I (Promega) at 37°C for 30 min, extracted with phenol/chloroform twice and chloroform once, and precipitated with ethanol. After suspension in DEPC-treated dH_2O, 0.4 μg of RNA was incubated with 40 U of M-MuLV reverse transcriptase (Boehringer), 40 U of RNase inhibitor (Boehringer) and 4 μl of 10 μM T12MA, T12MC, T12MG or T12MT primer as described before (Liang and Pardee, 1992; Liang *et al.*, 1993) in 40 μl of RT buffer (25 mM Tris, pH 8.3, 37.6 mM KCl, 1.5 mM $MgCl_2$, 5 mM DTT, 20 μM dNTP) at 37°C for 1 h. After heat inactivation of the reverse transcriptase at 95°C for 5 min, 2 μl of the sample was used for PCR in 20 μl of PCR buffer (10 mM Tris, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin and 2 μM dNTP) with 2 μl of 20 μM arbitrary primer (Liang and Pardee, 1992; Liang *et al.*, 1993) and 2 μl of 10 μM T12MA, T12MC, T12MG or T12MT primer, 1 μl of [α-^{35}S]dATP (NEN, 1200 Ci/mmol) and 0.3 μl of Taq polymerase (Boehringer). The parameters for PCR were as follows; 40 cycles of cycling step (94°C for 15 s, 40°C for 2 min, 72°C for 20 s) followed by 72°C elongation step for 5 min (Geneamp PCR system 9600, Perkin–Elmer Cetus). The amplified cDNAs were separated on a 6% sequencing gel. The positive bands were excised from the dried gel, boiled in 100 μl of dH_2O for 15 min, precipitated with ethanol and suspended in 10 μl of dH_2O. Four μl of recovered cDNAs were used for re-amplification by PCR in 40 μl of reaction volume with the same buffer and primers used for initial amplification except a higher concentration of dNTP (20 μM). The parameters for PCR were the same as in the initial amplification except that fewer cycling steps were used (30 cycles). Re-amplified cDNAs were filled-in with Klenow enzyme, separated on an agarose gel, recovered from the gel and ligated into pCRscript (Stratagene), and used for sequencing. cDNAs were isolated from plasmids and used as probes for Northern blot analysis and library screening.

Northern blot analysis

Total RNAs were extracted from cells with RNAzolB (TEL-TEST, INC) and chloroform, and precipitated with isopropanol. RNAs were separated on 1% MOPS−formaldehyde gel and transferred to hybond-N+ (Amersham). After prehybridization and hybridization, the filters were washed in 0.2× SSC/0.1% SDS at 55°C.

Library screening

To identify full length cyclin G cDNA clones, 5×10^5 recombinant plaques from a mouse embryonic stem cell cDNA library were screened. A cyclin G cDNA clone obtained by differential screening was used as a probe. Prehybridization and hybridization were performed as decribed (Sambrook *et al.*, 1989). Hybridized nylon filters were washed in 0.2× SSC/0.1% SDS at 55°C.

To identify cyclin G genomic clones, 5×10^5 recombinant plaques from a mouse genomic library were screened. A full length mouse cyclin G cDNA was used as a probe. Prehybridization and hybridization were performed as decribed (Sambrook *et al.*, 1989). Hybridized nylon filters were washed in 0.1× SSC/0.1% SDS at 55°C.

DNA binding assay

The immunoprecipitation assay was performed essentially as described previously (El-Deiry *et al.*, 1992). In order to identify the positive band,

phage DNA containing the genomic cyclin G gene (~47 kb in size) was digested with *Hin*fI and end-labeled with Klenow enzyme. End-labeled phage DNA (10 ng) was incubated with 5 μl of lysates prepared from Sf9 cells expressing p53, 0.5 μg of poly dI-dC and 0.4 μg of affinity-purified PAb421 at 4°C for 30 min in 100 μl of IP buffer (20 mM Tris, pH 7.2, 100 mM NaCl, 1% NP-40, 10% glycerol, 5 mM EDTA and 5 mM DTT). The DNA–p53–PAb421 complex was incubated with 40 μl of IP buffer containing 10 μg of poly(dI-dC) and 15 μl of protein A–sepharose at 4°C for 30 min, precipitated and washed four times with IP buffer. The precipitate was incubated with 200 μg/ml protein K and 0.5% SDS at 50°C for 1 h, and the supernatant was extracted with phenol and chloroform, precipitated with ethanol and separated on a 10% non-denaturing polyacrylamide gel.

In order to clone the positive band (HH0.34), phage DNA containing the genomic cyclin G gene was digested with *Eco*RI and *Sal*I, and the digested fragments were isolated from an agarose gel. Each isolated fragment was then digested with *Hin*fI, end-labeled and used for an immunoprecipitation assay to identify the fragment that contains HH0.34. An immunoprecipitation assay was done as described above. An *Eco*RI–*Sal*I 8.0 kb genomic fragment was found to contain HH0.34. This fragment was subcloned into pBluescript, digested with *Hin*fI, and the band corresponding to HH0.34 was subcloned into pBluescript.

For a gel shift assay, 1 ng of end-labeled oligonucleotide probe was incubated with 1.2 μl of lysate prepared from Sf9 cells expressing p53, 60 ng of poly(dI-dC), 1.2 μl of PAb421 (tissue culture supernatants) and various amounts of competitors on ice for 30 min in 12 μl of IP buffer. The products were separated on 4% non-denaturing polyacrylamide gels. WT30 and MUT30 were made by annealing two oligonucleotides (GATCTGCCAAGGCTTGCCCGGGCAGGTCTGGCCT and GATCAGGCCAGACCTGCCCGGGCAAGCCTTGGCA for WT30, GATCTGCCAAGGATTTCCCGGTCAGGTCTGGCCT and GATCAGGCCAGACCTGACCGGGAAATCCTTGGCA for MUT30).

Plasmid construction

To construct ptk-luc, pBLcat2 (Lucklow and Schutz, 1987) was digested with *Hin*dIII, blunt-ended with Klenow fragment, digested with *Bgl*II, and the fragment containing the HSV-tk promoter was ligated into pGL2Basic (Promega) which was digested with *Mlu*I, blunt-ended by Klenow fragment and digested with *Bgl*II. The 0.34 kb *Hin*fI–*Hin*fI fragment from the genomic cyclin G clone containing the p53 binding site (HH0.34) was blunt-ended with Klenow fragment, and cloned into the *Sma*I site of pBluescriptKS to generate pKS-GGHH0.34. To construct pHH0.34-tk-luc, pKS-GGHH0.34 was digested with *Eco*RV and *Sac*I, and the isolated genomic fragment was ligated into ptk-luc digested with *Sma*I and *Sac*I. WT30 was phosphorylated with polynucleotide kinase, and ligated into the *Bam*HI site of pBluescriptKS to generate pKS-WT30. To construct pWT30-tk-luc, pKS-WT30 was digested with *Eco*RV and *Sac*I, and ligated into ptk-luc digested with *Eco*RV and *Sac*I. pKS-MUT30 and pMUT30-tk-luc were constructed in the same manner as pKS-WT30 and P-WT30-tk-luc, respectively.

Transient transfection assay

For calcium phosphate transient transfection, 2 μg of effector construct, 5 μg of reporter contruct, 2 μg of pSV-βgal and 21 μg of pBluescript were used. Following the glycerol shock treatment, cells were harvested 48 h after transfection. The averages of two independent experiments are shown in Figure 4D.

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References

Baker,S.J., Markowitz,S., Fearon,E.R., Willson,J.K.V. and Vogelstein,B. (1990) *Science*, **249**, 912–915.

Bueno,A., Richardson,H., Reed,S.I. and Russel,P. (1991) *Cell*, **66**, 149–159.

Chapman,D.L. and Wolgemuth,D.J. (1992) *Mol. Reprod. Dev.*, **33**, 259–269.

Connolly,T. and Beach,D. (1994) *Mol. Cell. Biol.*, **14**, 768–776.

Coverley,D. *et al.* (1991) *Nature*, **349**, 538–541.

Coverley,D., Kenny,M.K., Lane,D.P. and Wood,R.D. (1992) *Nucleic Acids Res.*, **20**, 3873–3880.

Diller,L. *et al.* (1990) *Mol. Cell. Biol.*, **10**, 5772–5781.

Donehower,L.A. and Bradley,A. (1993) *Biochim. Biophys. Acta*, **1155**, 181–205.

Dutta,A. and Stillman,B. (1992) *EMBO J.*, **11**, 2189–2199.

El-Deiry,W.S. *et al.* (1993) *Cell*, **75**, 817–825.

El-Deiry,W.S., Kern,S.E., Pietenpol,J.A., Kinzler,K.W. and Vogelstein,B. (1992) *Nature Genet.*, **1**, 45–49.

Fotedar,R. and Roberts,J.M. (1992) *EMBO J.*, **11**, 2177–2187.

Gu,Y., Turck,C.W. and Morgan,D.O. (1993) *Nature*, **366**, 707–710.

Harper,J.W., Adami,G.R., Wei,N., Keyomarsi,K. and Elledge,S.J. (1993) *Cell*, **75**, 805–816.

Hartwell,L. (1992) *Cell*, **71**, 543–546.

Juven,T., Barak,Y., Zauberman,A., George,D.L. and Oren,M. (1993) *Oncogene*, **8**, 3411–3416.

Kastan,M.B. *et al.* (1992) *Cell*, **71**, 587–597.

Kastan,M.B., Onyekwere,O., Sidransky,D., Vogelstein,B. and Craig,R.W. (1991) *Cancer Res.*, **51**, 6304–6311.

Lane,D.P. (1992) *Nature*, **358**, 15–16.

Levine,A.J., Momand,J. and Finlay,C.A. (1991) *Nature*, **351**, 453–456.

Levy,N., Yonish-Rouach,E., Oren,M. and Kimchi,A. (1993) *Mol. Cell. Biol.*, **13**, 7942–7952.

Liang,P. and Pardee,A.B. (1992) *Science*, **257**, 967–971.

Liang,P., Averboukh,L. and Pardee,A.B. (1993) *Nucleic Acids Res.*, **21**, 3269–3275.

Lowe,S.W., Ruley,H.E., Jacks,T. and Housman,D.E. (1993) *Cell*, **74**, 957–967.

Lucklow,B. and Schutz,G. (1987) *Nucleic Acids Res.*, **15**, 5490.

Martinez,J., Georgoff,I., Martinez,J. and Levine,A.J. (1991) *Genes Dev.*, **5**, 151–159.

Nelson,W.G. and Kastan,M.B. (1994) *Mol. Cell. Biol.*, **14**, 1815–1823.

Prelich,G., Kostsura,M., Marshak,D.R.,Mathews,M.B. and Stillman,B. (1987) *Nature*, **326**, 471–475.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning. A Laboratory Manual.* 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Shi *et al.* (1994) *Science*, **263**, 1143–1145.

Shivji,K.K., Kenny,M.K. and Wood,R.D. (1992) *Cell*, **69**, 367–374.

Tamura,K. *et al.* (1993) *Oncogene*, **8**, 2113–2118.

Wu,X., Bayle,J.H., Olson,D. and Levine,A.J. (1993) *Genes Dev.*, **7**, 1126–1132.

Xiong,Y. *et al.* (1993) *Nature*, **366**, 701–704.

Yonish-Rouach,E. *et al.* (1991) *Nature*, **352**, 345–347.

Yonish-Rouach,E. *et al.* (1993) *Mol. Cell. Biol.*, **13**, 1415–1423.

Zambetti,G.P., Bargonetti,J., Walker,K., Prives,C. and Levine,A.J. (1992) *Genes Dev.*, **6**, 1143–1152.

Zhu,L. *et al.* (1993) *Genes Dev.*, **7**, 1111–1125.

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