p21/CDKN1A mediates negative regulation of transcription by p53

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SUMMARY

The tumor suppressor p53 regulates transcription positively and negatively, depending on the target gene. Whereas p53 induces transcription through direct interaction with promoter DNA, the mechanism of p53-mediated transcriptional repression is less well understood. Early reports described the alleviation of p53-mediated repression by inhibitors of apoptosis, suggesting that negative regulation of transcription might occur only in conjunction with programmed cell death. More recently, it has been proposed that certain genes, such as survivin, are repressed by direct association of p53 with their promoters, followed by recruitment of a repressor complex. We show here that p53-mediated negative regulation of transcription could occur independently of apoptosis. In contrast, the aminoterminal transactivation domain of p53 was required for negative regulation of transcription. Similarly, the p53-homologue p73 diminished the expression of survivin and stathmin, depending on its transactivation domain. Mutation of the putative p53 binding site within the survivin promoter did not impair its repression. These observations raised the hypothesis that activation of an effector gene might be required for repression by p53. Strikingly, when the p53-inducible p21/CDKN1A gene was deleted, p53 no longer repressed any one among 11 genes that it downregulates otherwise. Most of these genes were also repressed by ectopic p21 in the absence of p53. Overexpressed c-MYC reduced the transcription of p21/CDKN1A and impaired p53-mediated repression, but did not abolish repression by ectopic p21. Taken together, these results strongly suggest that increased expression of p21/CDKN1A is necessary and sufficient for the negative regulation of gene expression by p53.
INTRODUCTION

p53 is a key regulator of cell growth and apoptosis. Its central role in tumor suppression becomes evident by the fact that the \(p53\) gene is mutated in about 50% of human malignancies. p53 acts as a transcription factor, modulating the expression of growth and death regulators. As a result, cell proliferation is suppressed and/or programmed cell death is induced (1). It is generally accepted that p53 activates a number of promoters through direct interaction with the promoter DNA and the subsequent recruitment of the basal transcription machinery, \(e.\, g.\) the TFIID complex and the p300/CPB histone acetyl transferases. A tetramer of p53 molecules is assembled through the carboxyterminal oligomerization domains. This allows the central domains to interact directly with a consensus DNA element. As a consequence, the aminoterminal transactivation domains interact with basal transcription factors, resulting in increased gene expression (2,3).

However, it has long been noticed that some genes are negatively regulated (referred to as “repressed” hereafter) by p53, and the list of those genes has been extended for almost a decade (Table 1). When analyzing p53 mutants, and p53 in combination with inhibitors of apoptosis, a striking correlation of p53-mediated transcriptional repression and p53-induced apoptosis was observed, and this raised the hypothesis that negative regulation of gene expression by p53 might be crucial for the induction of apoptosis (4-7).

In contrast to p53-mediated gene activation, the mechanism(s) of transcriptional downregulation of genes by p53 remain controversial. Basically, three different scenarios can be envisioned:

a) Negative regulation of gene expression by p53 might merely represent an epiphenomenon of apoptosis, \(i.\, e.\) p53 downregulates certain genes not directly but only through the onset of cell death. This model is supported by the observed correlation between the ability of p53 mutants to repress transcription in reporter assays, and their potential to induce apoptosis. Such a
correlation exists in the case of p53 mutants lacking the proline-rich domain within residues 62-91 (8,9) or point mutants at residues 175 (10) and 246 (8). Further, early reports describe the downregulation of reporter gene expression by p53, which was found to be reverted by inhibitors of apoptosis (4,5). However, it should be noticed that in transient reporter assays, a large variety of promoters can be found “repressed” by p53 (5), and that this may not necessarily reflect the regulation of the corresponding cellular genes in all cases.

b) Alternatively, p53 may lead to the negative regulation of gene expression by virtue of a repressor function, and by direct interaction with promoter DNA. Such a scenario has been proposed for the repression of *stathmin* and *survivin* by p53 (11,12). p53 was found to interact with the mSin3a protein that is part of transcriptional repressor complex (11). Further, at least in the case of *survivin*, a p53 binding sequence was reported to exist within the promoter (12). However, the question remains how p53 would simultaneously activate some genes and repress other genes after binding to the respective promoters. Further, if p53 activates and represses by different mechanisms, these functions could be expected to be separable by mutational analysis. However, no p53 mutant has been described that represses but no longer transactivates. Instead, a mutation at residues 22/23 abolishes both transactivation and repression (7,13), although the interaction of p53 with mSin3a was mapped to amino acids 61-75 within p53 (14). Unless a clear mode of distinction between activated and repressed promoters could be defined, the plausibility of a direct repression model remains to be questioned.

c) A third model that will be proposed in this work ascribes p53-mediated repression to the induction of a repressor. Thus, p53 would first increase the levels of another transcriptional regulator, which in turn would negatively affect the expression of downstream genes. The first p53-induced gene to be identified was *p21/Cip1/waf1/CDKN1A* (15). As an inhibitor of
cyclin dependent kinases (cdks), p21 is known to prevent the phosphorylation of retinoblastoma (Rb) family proteins and hence lead to the accumulation of hypophosphorylated pRb (16). This protein species, in turn, binds to E2F family transcription factors and converts them from transcriptional activators to transcriptional repressors. Thus, it appears conceivable that p53 may negatively regulate the expression of genes through the induction of p21/CDKN1A and the consecutive hypophosphorylation of pRb and its relatives. Indeed, certain cell cycle regulators responsible for G2 arrest, such as CHK1 (17) and cdc2 (18,19), were found to be downregulated at the mRNA and proteins levels by p53 and the p21/pRb/E2F pathway. However, the general dependence of p53-mediated negative gene regulation on the expression of p21 remains to be assessed.

This study was aiming at the distinction between these three models. First, we provide evidence that, at least in the cases of survivin and stathmin, negative gene regulation by p53 is not a consequence of apoptosis. Secondly, p53 and the p53-homologue p73 were capable of downregulating the expression of these genes, but each required a transactivation domain to do so. Analysis of the survivin promoter suggested that sequence elements other than the putative p53 binding motif are needed for repression by p53. Most strikingly, however, we show that the negative regulation of all p53-repressed genes analyzed entirely depends on the presence of p21/CDKN1A, and that overexpressed p21 represses a similar set of genes. These results strongly argue that p53-mediated repression occurs mainly through the induction of the p21/CDKN1A gene.
EXPERIMENTAL PROCEDURES

Cell culture and transfections. H1299 cells (p53 -/-) were maintained in Dulbecco’s modified eagle medium (DMEM; Life Technologies) with 10% fetal bovine serum (FBS). HCT116 cells with and without mutations disrupting p53 and p21/CDKN1A (20) were kindly provided by K. Roemer with generous permission by B. Vogelstein, and were cultivated in McCoy’s medium (Life Technologies) with 10% FCS. Transfections were done using FuGene6 (Roche). Doxorubicin (Sigma) was added to the cell culture media where indicated at a concentration of 350 nM for 48 hours. zVAD (Calbiochem) was used at 100 µM.

Plasmids and adenovirus vectors. A reporter construct containing the survivin promoter (21) was kindly provided by D. C. Altieri. Mutants of this promoter were created in this plasmid background by the QuikChange methodology (Stratagene) for site directed mutagenesis, using the following primers and their respective reverse complements:

mutant 1: GAG GGC GTG CGC TCC CGG GAT GCC CCG CGC GCC; mutant 2: CTA AGA GGG CGT ACG CTC CCG ACA TG; mutant 3: CTC CCG TGC CCA TGG CGC GCC ATT AAC. Mutant 4 was obtained by sequential mutagenesis using the oligonucleotides corresponding to mutant 2 and mutant 3. All plasmids were confirmed by sequencing.

The expression plasmid pRcCMVp53 (13) was used in reporter assays.

Adenovirus vectors expressing p53 and β-galactosidase have been described (22), as well as adenovirus vectors to express p73ΔN proteins (23) and a similar vector to express c-MYC (24). An adenovirus expression vector for p21 (25) was originally obtained from G. Nabel and provided to us by R. Iggo.
An adenovirus vector to express p73TAβ was generated using the AdEasy system (26). The p73TAβ coding region (without HA tag) together with the 5’ untranslated region of human lamin mRNA was excised with HindIII and XbaI from the corresponding pcDNA3-based plasmid (27), and introduced into the same sites of the shuttle vector pAdTrack-CMV. This plasmid was allowed to recombine with the vector pAdEasy1, followed by treatment with PacI and transfection into an E1-complementing cell line. An adenovirus vector to overexpress adenovirus E1B-19 kDa was generated similarly. In this case, the E1B-19kDa coding region was excised with HindIII and EcoRI from the corresponding pcDNA3-based plasmid (4), filled in with Pfu DNA polymerase (Stratagene) and introduced into the EcoRV sites of the shuttle vector pAdTrack-CMV, followed by recombination with pAdEasy and transfection. An adenovirus vector to express p53mt22/23, with the mutation L22Q/W23S, was created by cloning the corresponding coding region from pRcCMVp53mt22/23 (13) into pAdtrackCMV using HindIII and XbaI, followed by recombination and transfection. Viruses were amplified, the titer was determined and infections were carried out as described (22,28).

Immunoblot. Proteins were separated on SDS polyacrylamide gels and transferred to nitrocellulose, followed by incubation with antibodies in PBS containing 5% milk powder and 0.05% Tween 20. Peroxidase-coupled secondary antibodies (whole immunoglobulin G, Jackson) were then detected by chemiluminescence (Pierce). Antibody Pab1801 to p53, antibody Ab-1 to p21/cip1/waf1 and antibody Ab-2 against poly(ADP-ribose) polymerase (PARP) were from Calbiochem. Another monoclonal mouse antibody against actin (clone C-2) was from Santa Cruz.

Semiquantitative RT-PCR. H1299 cells were transduced with adenovirus vectors, and HCT116 cells were treated with doxorubicin. After 48 hours, total RNA was prepared (Trizol Reagent, Life Technologies), followed by reverse transcription with Superscript II polymerase (Life
Technologies) and PCR amplification with Expand HiFi DNA polymerase (Roche). The PCR temperatures consisted of a 3 minutes denaturation step at 96 °C, followed by the indicated numbers of cycles at 96 °C for 30 s, 57 °C for 30 s and 70 °C for 50 s. These temperature cycles were used for all amplifications, except that in the case of *ubiquitin*, the 57 °C step was omitted. The primers and numbers of PCR cycles used were as follows. For each gene, the reverse transcription was started with the first (RT) oligonucleotide, whereas the PCR was carried out using the second (forward) and third (reverse) oligonucleotide.

**BRCA1**: 30 cycles, RT: oligo dT, forward: CCA AAG CGA GCA AGA GAA TCC CAG, reverse: TCA GGT AGG TGT CCA GCT CCT GGC.

**CDC2**: 25 cycles, RT: oligo dT, forward: CCT TGC CAG AGC TTT TGG AAT ACC, reverse: GAC ATG GGA TGC TAG GCT TCC TGG.

**CDC25C**: 25 cycles, RT: oligo dT, forward: GTA TCT GGG AGG ACA CAT CCA GGG, reverse: CAA GTT GGT AGC CTG TTG GTT TG.

**CHEK1**: 30 cycles, RT: oligo dT, forward: CCT TTG TGG AAG ACT GGG ACT TGG, reverse: CAT CTT GTT CAA CAA ACG CTC ACG.

**Cyclin A2**: 25 cycles, RT: oligo dT, forward: AGC AGC CTG CAA ACT GCA AAG TTG, reverse: TGG TGG GTT GAG GAG AGA AAC ACC.

**Cyclin B1**: 30 cycles, RT: oligo dT, forward: CCT CTA CCT TTG CAC TTC CTT CGG, reverse: GAG TGC TGA TCT TAG CAT GCT TCG.

**LBR**: 25 cycles, RT: oligo dT, forward: TGG CTT TTG GAG ACT TGG TGT GGG, reverse: ATC ACC CAA GTA ATT GGG GTG GCG.
p21 / CDKN1A: 30 cycles, RT: GGA AAA GGA GAA CAC GGG ATG AGG AGG, forward: CCT GGC ACC TCA CCT GCT CTG CTG, reverse: GCA GAA GAT GTA GAG CGG GCC TTT.

PIG3: 30 cycles in Fig. 3, 25 cycles in all other cases, RT: CGG TGA GCA GGC CTC TGG GAT GGC, forward: GTG CAC TTT GAC AAG CCG GGA GGA, reverse: CAG CCT GGG TCA GGG TCA ATC CCT.

POLD1: 30 cycles, RT: oligo dT, forward: GCC GCA CAG TGC TCA GCC ACC AGG; reverse: CGC ATG TAG AAG ATG GGG CAG TCC.

Stathmin: 25 cycles, RT: CCT TCT GAA GCA CTT CTT C, forward: GTG GTC AGG CGG CTC GGA CTG, reverse: CTC TCG TTT CTC AGC CAG CTG C.

Survivin: 25 cycles, RT: CAG AGG CCT CAA TCC ATG GCA GCC, forward: GGC AGC CCT TTC TCA AGG ACC ACC, reverse: GAT GGC ACG GCG CAC TTT CTT CGC.

Top 2 alpha: 25 cycles, RT: oligo dT, forward: TGT CGT GTC AGA CCT TGA AGC TG, reverse: CCT TGG ATT TCT TGC TTG TGA CTG.

Ubiquitin (UbC): 25 cycles, RT: ACT GGG CTC CAC CTC AAG GGT GAT, forward: GTC GCA GCC GGG ATT TGG GTC GCG, reverse: GTC TTG CCA GTG AGT GTC TTC ACG.

Electrophoretic mobility shift assay (EMSA). The interaction of p53 with promoter elements was analyzed as described (8,23,29). We generated p53 and its mutants by in vitro transcription and translation using T7 RNA polymerase and rabbit reticulocyte lysate (Promega). These preparations were incubated with 1 µl sheared salmon sperm DNA (0.1 µg/µl), 1 µl poly dAdT (1 µg/µl), 7 µl EMSA buffer (25 mM Tris-Cl pH 7.5, 130 mM NaCl, 3 mM KCl, 5% bovine serum albumine, 12% glycerol, 1 mM dithiothreitol) and, when indicated, 1 µl...
monoclonal antibody 421 against p53 (Calbiochem; 100 µg/ml), for 10 minutes at 23 °C. Subsequently, we added 1 ng of the radiolabelled DNA probe and continued the incubation for 1 hour at 23°C. The reaction mixes were then separated at 4 °C on a native 5% polyacrylamide gel with 0.5 TBE as running buffer, followed by autoradiography using a Bioimager (Fuji). We generated the probes by annealing the oligonucleotides indicated below to each other and performing fill-in reactions (exo− Klenow enzyme, MBI Fermentas) including α-32P-dCTP. The oligonucleotides had the following sequences:

survivin forward: GAC CGC CTA AGA GGG CGT GCG CTC CCG AC

survivin reverse: GGG CAT GTC GGG AGC GCA CGC CCT CTT AGG C

mutant survivin forward: GAC CGC CTA AGA GGG CGT GCG CTC CCG GG

mutant survivin reverse: GGG CAT CCC GGG AGC GCA CGC CCT CTT AGG C

p21 forward: GAT CGC GGC CGC GAA CAT GTC CCA ACA

p21 reverse: GGG CAA CAT GTT GGG ACA TGT TCG CGG CCG C

mutant p21 forward: GAT CGC GGC CGC GAA AAT TTC CCA AAA

mutant p21 reverse: GGG CAA AAT TTT GGG AAA TTT TCG CGG CCG C
RESULTS

p53-mediated repression is not inhibited by a viral antagonist of pro-apoptotic mitochondrial factors.

We tested whether p53-mediated transcriptional repression might be a result of p53-induced apoptosis. p53 was expressed in the p53 -/- cell line H1299 using an adenovirus expression vector. To block apoptosis, the adenovirus E1B-19 kDa protein was co-expressed with p53. E1B-19 kDa interferes with the pro-apoptotic functions of the mitochondrial bax and bak proteins (30) and inhibits p53-mediated cell death (31). Apoptosis was assessed by immunoblot detection of poly (ADP ribose) polymerase (PARP). Cleavage of PARP, a characteristic of apoptotic cells (32), was detected when p53 was expressed alone but not when p53 and E1B-19 kDa were co-expressed (Fig. 1A). In a parallel experiment, identically treated cells were harvested to prepare RNA, and the levels of survivin and stathmin mRNA were determined by reverse transcription and semiquantitative polymerase chain reaction (RT-PCR). Both genes were clearly downregulated by p53, regardless of the presence or absence of E1B-19 kDa (Fig. 1B). The expression of a control gene, ubiquitin C, remained unchanged, and the transactivation of a p53-responsive gene, PIG3, was not affected by E1B-19 kDa. Finally, the repression of the survivin promoter by p53 in luciferase reporter assays was not detectably influenced by E1B-19 kDa (Fig. 1C). We concluded that p53-mediated negative regulation of transcription occurs independently of apoptosis.

p53-mediated negative regulation is not inhibited by a synthetic inhibitor of caspases.

A similar set of experiments was used to determine whether caspase inhibition would affect p53-mediated transcriptional repression. The peptide caspase inhibitor zVAD (33) effectively prevented p53-induced apoptosis, as determined by PARP detection (Fig. 2A), but did not affect
the capability of p53 to repress the survivin and stathmin genes, as revealed by RT-PCR (Fig. 2B). Thus, p53-induced apoptosis can be prevented by caspase inhibition without compromising its ability to downregulate gene expression.

**p53 requires a functional transactivation domain to mediate negative regulation of gene expression.**

To test whether the aminoterminal transactivation domain of p53 is necessary for the negative regulation of gene expression, we transduced H1299 cells to express p53, with or without a mutation near the aminoterminal (L22Q/W23S) that abolishes the function of the transactivation domain (13). Unlike wild type p53, the mutant was not only defective with regard to the induction of PIG3 but also lost the capability of repressing the expression of survivin and stathmin, as determined by semiquantitative RT-PCR (Fig. 3). Thus, as in other assay systems (7,13), the negative regulation of gene expression by p53 requires a functional transactivation domain.

**p73 negatively regulates transcription similarly to p53, depending on a transactivation domain.**

Next, we asked whether the p53-homologue p73 can downregulate the transcription of p53-repressible genes. We expressed the p73TAβ isoform (Fig. 4A) in H1299 cells and determined the levels of survivin and stathmin mRNA. p73 and p53 were equally capable of downregulating survivin, and both reduced the expression of stathmin, albeit to a lesser extent in the case of p73 (Fig. 4B). Naturally occurring p73 isoforms that lack the transactivation domain (p73ΔNα and p73ΔNβ) were analyzed in parallel. These isoforms did not repress. We conclude that p73 has a similar capability of downregulating gene expression as p53, but that a transactivation domain is required to carry out this function in both cases.
A p53 binding consensus element within the *survivin* promoter interacts inefficiently with p53, and binding is further impaired by mutation

The repression of the *survivin* promoter by p53 was previously suggested to be mediated by a direct interaction of p53 with a p53 binding consensus element (12). As a first step to understand the function of this element, we compared its capability of binding p53 with an established p53-responsive element derived from the *p21/CDKN1A* promoter, using electrophoretic mobility shift assay (EMSA). In each case, a mutated sequence element was used as a negative control. A comparison of these sequences with the previously defined p53-binding consensus (34,35) is shown in Fig. 5A. When p53 alone was assayed for binding the different DNA elements, only the wild type *p21/CDKN1A* sequence yielded a band with decreased electrophoretic mobility, indicating a DNA-protein interaction (Fig. 5B, compare lanes 1 and 2). The interaction was strongly increased by the monoclonal antibody 421 against the carboxyterminal portion of p53, resulting in a supershifted and much more intense signal (Fig. 5B, compare lanes 9 and 10), as described previously (36). Under these circumstances, the wild type sequence derived from the *survivin* promoter was also found to interact with p53, albeit with far lower efficiency than the *p21/CDKN1A* element (Fig. 5B, compare lanes 13 and 14). Even in the presence of antibody 421, the mutant sequence elements were not found to interact with p53 (Fig. 5B, lanes 11, 12, 15, and 16). Thus, the p53-binding consensus element of the *survivin* promoter can interact with p53 in a specific, mutation-sensitive manner, but with comparatively low efficiency, at least when assayed by EMSA.
A p53-binding consensus element within the *survivin* promoter is dispensable for p53-mediated repression.

The fact that a transactivation domain is needed to downregulate *survivin* expression by p53 prompted us to analyze the *survivin* promoter with regard to its negative regulation. It was previously suggested that p53 may directly interact with the promoter DNA through a consensus p53 binding element (12), and such an interaction was observed *in vitro* (Fig. 5). However, it should be noted that this promoter element is not conserved when comparing the human and the murine sequence (for alignment of the promoter sequences, cf. (21)). The previously described p53 binding motif within the human *survivin* promoter (12) was mutated as in Fig. 5, at the site that is most conserved among p53 binding sequences, namely, the C residue within a RRRCW (R = purine, W = A or T) half site of the p53-responsive consensus element (34,35). Moreover, mutations within two putative CDE consensus motifs were introduced into the luciferase reporter construct, alone or in combination (Fig. 6A). These constructs were then tested for reporter expression in the presence or absence of a p53 expression plasmid (Fig. 6, B and C). It was found that mutations in the CDE-like motifs resulted in reduced reporter expression, but alleviated or abolished further downregulation by p53. In contrast, when the putative p53 binding site was mutated, the promoter strength and susceptibility to repression by p53 essentially remained as in the wild type promoter. We conclude that the putative p53 binding site is not required to confer p53-mediated repression to the *survivin* promoter. Thus, the proposed direct binding by p53 (12) does not provide an appropriate explanation for the observed repression.

**p53-mediated negative regulation is dependent on the expression of p21/CDKN1A**

The requirement of a transactivation domain to repress *survivin* and *stathmin* prompted us to test whether a p53-induced gene product might mediate repression. A candidate for such a mediator
gene was \textit{p21/CDKN1A}, since the product of this p53-responsive gene can be expected to repress genes by hypophosphorylation of Rb family members, and the conversion of E2F transactivators into repressors (16). Therefore, we induced p53 accumulation and activity by treating HCT116 cells with doxorubicin, as verified by immunoblot detection of p53 and p21 (Fig. 7A, lanes 1 and 2). In addition, cells of the same line were used that carry targeted disruptions of the \textit{p21/CDKN1A} and \textit{p53} genes (20) (Fig. 7A, lanes 3-6). All three cell lines were treated and analyzed in parallel. Eleven genes that were previously reported to be repressed by p53 (cf. Table 1) were found downregulated in doxorubicin-treated wild type HCT116 cells (Fig. 7B). The known p53-inducible gene \textit{PIG3} (29,37) was activated. Both repression and activation were defective in \textit{p53 -/-} cells, as expected. Surprisingly, however, in cells lacking \textit{p21/CDKN1A}, p53 was no longer capable of downregulating the expression of any gene that was repressed in wild type cells, while still being able to induce \textit{PIG3}. Hence, expression of \textit{p21/CDKN1A} is mandatory to allow p53-mediated negative regulation of all of these genes.

\textbf{\textit{p21/CDKN1A} is sufficient for repression}

The results described above indicate that p21 is required for repression of transcription by p53. This raised the question whether the enhanced expression of p21 might be sufficient for this effect, even in the absence of p53. To test this, p21 was overexpressed in H1299 cells using an adenovirus vector. For comparison, p53 and \textit{\beta}-galactosidase were expressed in parallel experiments. The amounts of p21 were comparable when endogenous expression was induced by p53, or when exogenous p21 was expressed by the virus, as determined by immunoblot (Fig. 8A). Subsequently, the mRNA levels of p53-respressible genes were analyzed (Fig. 8B). In most cases, p21 alone was sufficient to repress these mRNA levels to an extent comparable with the effect of p53. In some cases (\textit{e. g. stathmin}, cf. Fig. 8B), however, repression was less pronounced in the presence of exogenous p21, when compared to p53. In another case, the
mRNA levels of LBR were not reduced by p53 nor by p21, whereas they were repressed in HCT116 cells (cf. Fig. 7B), arguing that the susceptibility of this gene to repression depends on the cell type. Thus, in some cases, p53 appears to employ cellular factors other than p21 to efficiently repress gene expression. Nonetheless, p21 is not only uniformly required for repression but also sufficient for the negative regulation of the majority of genes under study here. We conclude that p21 is a principal mediator of repression by p53.

**c-MYC impairs the repression of survivin and stathmin by p53.**

The c-MYC protein was recently found to suppress the induction of p21/CDKN1A by p53 (38,39). Since p21/CDKN1A is required for negative gene regulation by p53, we reasoned that c-MYC might interfere with p53-mediated repression. To test this, c-MYC and p53 were expressed by adenovirus vectors in different combinations, followed by assessment of the mRNA levels of p21/CDKN1A, survivin and stathmin. As shown in Fig. 9A, c-MYC not only reduced the amount of p21/CDKN1A mRNA, in agreement with previous reports, but also alleviated the repression of survivin and stathmin. Hence, c-MYC antagonizes p53-mediated transcriptional repression. Based on these findings, we hypothesized that the block of p21/CDKN1A expression by c-MYC may be the reason why c-MYC inhibits p53-mediated repression. To test this, we assessed the effect of c-MYC on repression by overexpressed p21. As shown in Fig. 9B, c-MYC did not abolish repression of survivin and stathmin by p21. We conclude that the negative regulation of p21/CDKN1A by c-MYC is a pre-requisite for the ability of c-MYC to abolish the repression of survivin and stathmin.
DISCUSSION

Our results demonstrate that the induction of p21/CDKN1A is essential for p53-mediated negative regulation of transcription, whereas the onset of apoptosis is not. This strongly argues that p53 reduces the expression of certain genes indirectly by enhancing the expression of p21/CDKN1A. c-MYC negatively regulates the expression of p21/CDKN1A and also prevents p53-mediated repression.

It is tempting to speculate that p21 carries out its function in p53-mediated repression by inactivating cyclin dependent kinases. This may lead to the hypophosphorylation of retinoblastoma family proteins, and to the repression of E2F-responsive promoters. Retinoblastoma proteins can convert E2F proteins from transcriptional activators to transcriptional repressors (40). Strikingly, a large proportion of the known p53-repressed genes have also been reported to be regulated by E2F proteins, and in some cases, association of E2Fs with their promoters has been detected by chromatin immunoprecipitation or EMSA (Table 1). The concept that p53 converts E2Fs into transcriptional repressors through p21 is also supported by the finding that the p53-antagonist Mdm2 induces transactivation by E2F1 (41). Finally, a recent study shows that the repression of statmin in response to genotoxic stress can be inhibited by a dominant negative E2F mutant, as well as by the E7 protein from human papillomavirus type 16, which binds and inactivates Rb family proteins (42). Besides E2Fs, however, Rb family proteins may also affect the activity of other transcription factors, among them the ID2 protein (43). Possibly, Rb family proteins might also regulate CDE/CHR binding factors (44) or the transcription factor NF-Y that was shown to bind some p53-repressed promoters (45,46), and each of these factors could fail to activate transcription or even act as a transcriptional repressor in the presence of hypophosphorylated Rb family proteins. The dependence of a particular promoter on several of these factors might further increase its repression by p53 and p21. This
may explain why not all E2F-responsive promoters are equally susceptible to suppression by p53. Indeed, the first promoter (TA-promoter) of the p73 gene is strongly induced by E2F (47-49). Nonetheless, active p53 does not detectably influence the expression of p73TA (23). We propose that some promoters may be inducible by E2F while being only marginally susceptible to repression by the E2F/pRb complex, perhaps depending on the position of the E2F binding DNA element(s) and/or the activity of different transcription factors on these promoters.

Our analysis of the survivin promoter (Fig. 6) strongly suggests that no consensus p53 binding element is required to mediate repression. On the other hand, it was reported that removing the three spacer nucleotides between the two consensus sites of the putative p53 binding element turned transcriptional repression of this promoter into activation by p53. Therefore, it was suggested that the spacer nucleotides might render a p53 binding element susceptible to transcriptional repression by p53 (12). In contrast, it was proposed by others that p53 binding is not required to repress survivin transcription (50). Based on the results shown here (Fig. 6), we suggest that removing the spacer nucleotides in the previous report (12) newly created a p53-inducible element that overrides any repressing effect of p53 on the survivin promoter, whereas within the wild type sequence, the putative p53 binding site functions poorly and does not significantly alter transcription in any direction.

Since p53 induces apoptosis readily in cells lacking p21/CDKN1A (20,51), without repressing the genes analyzed (this study), we propose that repression is not a requirement for p53-induced apoptosis, at least in the systems studied here. This is in contrast to the previously suggested proapoptotic role of p53-mediated repression (4-7). Survivin was shown to inhibit apoptosis when overexpressed (50,52), and its synthesis is downregulated by p53 ((12) and this study). Nonetheless, our data suggest that downregulation of survivin is not required for the induction of
apoptosis. Apparently, p53 triggers apoptosis primarily through the induction of pro-apoptotic genes rather than the repression of anti-apoptotic genes.

p21 induces cell cycle arrest in the phases G1 and G2. p53-mediated G2-arrest does not occur in the absence of p21 (20). Consistently, the survivin gene was not only reported to inhibit apoptosis, but also to promote mitosis (53). We conclude that indirect negative regulation of genes, including survivin, by p53, may be required for the G2 arrest, rather than for the induction of apoptosis. Further, the fact that overexpression of cyclin B1 or a dominant active form of cdc2 can override p53-mediated G2 arrest (54) argues that repression of cdc2 and cyclin B1 by p53 through p21 may be necessary for p53-triggered arrest in G2.

If p53 can downregulate genes indirectly through the induction of an effector gene, similar mechanisms might apply to the regulation of certain p53-activated genes. A subset of p53-inducible genes may not be induced directly through binding of p53 to their promoters, but rather indirectly through the enhanced expression of a transcriptional regulator. An example may be represented by the induction of the insulin-like growth factor binding protein 3 gene by p53, since the product of this gene interferes with insulin-like growth factor signalling and the subsequent transcriptional regulation (55).

After its discovery, a plethora of activities exerted by p53 was described in vitro and in vivo, leading to an extremely complex picture and some confusion about which of these activities are essential for tumor suppression (56). Recent knock-in studies revealed that a germline mutation corresponding to the aminoterminal domain of p53 in mice yields a phenotype that apparently cannot be distinguished from p53 -/- animals (57,58). This would imply that functions of the aminoterminal domain are essential for all biologically relevant p53 activities. However, it was less clear what biochemical function of this domain, transactivation or repression, or both,
mediates these activities. The evidence presented here strongly suggests that repression by p53 occurs indirectly through the transcriptional activation of p21/CDKN1A. Thus, it remains possible that p53’s complex biological effects might all be a result of transactivation.

**ACKNOWLEDGEMENTS**

We thank H.-D. Klenk for continuous support. B. Vogelstein and K. Roemer for providing HCT116 cells and mutants. D. C. Altieri for the reporter construct containing the survivin promoter. B. Vogelstein and H. Hermeking for an adenovirus vector to express c-MYC. G. Nabel and R. Iggo for an adenovirus vector to express p21. K. Engeland for critically reading the manuscript and helpful suggestions. J. Roth and the members of our laboratory for stimulating discussions.

This work was supported by the Deutsche Forschungsgemeinschaft, the Wilhelm Sander-Stiftung, the Mildred Scheel Stiftung / Deutsche Krebshilfe, and the P. E. Kempkes Stiftung.
REFERENCES


22


*Oncogene* **18**(43), 5954-8.
LEGENDS TO FIGURES

Figure 1: Influence of adenovirus E1B-19 kDa on p53-mediated negative gene regulation

A. Inhibition of p53-mediated apoptosis by overexpression of adenovirus E1B-19 kDa

H1299 cells (p53 -/-) were transduced with adenovirus vectors to express p53 alone or together with E1B-19 kDa. A vector expressing beta-galactosidase was used as a negative control, as indicated. The multiplicity of infection was 20 for each virus. Twenty-four hours post infection, the cells were harvested and poly (ADP-ribose) polymerase was detected by immunoblot. A faster-running cleavage fragment is detected in apoptotic cells.

B. Influence of E1B-19 kDa on p53-mediated negative regulation of survivin and stathmin expression

The cells were treated identically, followed by RNA preparation and semiquantitative RT-PCR, detecting the indicated gene products.

C. Influence of E1B-19 kDa on p53-mediated negative regulation of the survivin promoter

H1299 cells were transfected with a luciferase reporter gene construct containing the survivin promoter (1 µg) and expression vectors for p53 (100 ng) and E1B-19 kDa (200 ng) as indicated, keeping the total amount of plasmid DNA at 1.5 µg by adding the empty vector plasmid pcDNA3 in each case. Twenty-four hours after transfection, luciferase activity was determined. The results were normalized with reference to the value obtained without co-expression of either protein. The average luciferase activities determined in at least three experiments are shown, together with the standard deviation.
**Figure 2: Influence of caspase activity on p53-mediated negative gene regulation**

A. **Inhibition of p53-mediated apoptosis by a peptide inhibitor of caspases**

H1299 cells were transduced to express p53 or beta-galactosidase using adenovirus vectors (multiplicity of infection = 20). Immediately after transduction, the peptide zVAD (100 µM) or the dimethylsulfoxazole (DMSO) solvent (1:1000 vol/vol) were added to the cells. After 24 hrs, poly (ADP-ribose) polymerase (PARP) cleavage was assessed as described in the legend to Fig. 1A. Note that in the presence of p53 and DMSO alone, neither full-length nor cleaved PARP was detected, presumably due to further degradation of the fragment. This was repeatedly observed when apoptosis had proceeded to a large extent, especially in the presence of DMSO.

B. **Influence of caspase activity on p53-mediated reduction of survivin and stathmin expression**

RNA was prepared and analyzed as described in the legend to Fig. 1B.

**Figure 3: Requirement of a transactivation domain for repression by p53**

H1299 cells were transduced to express p53, with wild type (wt) sequence or with mutations at residues 22 and 23 (p53mt22/23; mutation L22Q/W23S), followed by RNA preparation and RT-PCR analysis as described in the legend to Fig. 1B.

**Figure 4: Negative regulation of gene expression by p73 proteins**

A. **p73 isoforms in comparison to p53**

Homologous regions are depicted according to (59).

B. **mRNA levels in response to p53 and p73**

H1299 cells were transduced to express p53 or the indicated p73 isoforms, followed by RNA preparation and RT-PCR analysis (cf. legend to Fig. 1B).
Figure 5: A putative p53 binding element within the survivin promoter

A. p53-binding elements

A previously proposed p53-binding element (12) within the survivin promoter is depicted along with a mutant that was used in the experiment shown in B. A p53-responsive element from the p21/CDKN1A promoter and its mutant were analyzed in parallel. The previously defined p53-binding consensus element (34,35) is shown for comparison. Nucleotides not corresponding to the consensus are shown in lower case, and mutated nucleotides are underlined.

B. Electrophoretic mobility shift assays (EMSA)

Oligonucleotides corresponding to the sequence elements depicted in A. were employed to generate a radioactively labelled, double stranded DNA probe. This probe was incubated with reticulocyte lysates that had been programmed for translation in vitro with a plasmid encoding p53 (+), or with an “empty” vector plasmid (-). Subsequently, the mixtures were separated on a native polyacrylamide gel, followed by autoradiography. The monoclonal antibody 421 binding near the C terminus of p53 activates the cryptic p53 DNA binding activity (36). Therefore, this antibody was added to the reactions as indicated. The positions of complexes containing p53 with or without the antibody, as well as a background band that occurred independently of p53 (*), are indicated by arrows. Note that the free DNA was allowed to run out at the bottom of the gel, to increase the resolution of the different complexes.

Figure 6: Cooperation of several elements within the survivin promoter to allow p53-mediated repression

A. Mutations of the survivin promoter

A portion of the survivin promoter sequence is shown, with putative transcription factor binding sites indicated. The mutations introduced are shown below the wild type sequence.
B. Activity of *survivin* promoter mutants

A *survivin* promoter construct with or without the indicated mutations (1 µg each) was transfected into H1299 cells together with an empty pcDNA3 vector (500 ng), followed by luciferase assays, as described in the legend to Fig. 1C. The luciferase activity obtained with the wild type promoter was defined 100%, and the other values were normalized accordingly.

C. Requirement of promoter elements for p53-mediated repression

Each mutant of the *survivin* promoter construct was transfected together with increasing amounts of a p53 expression plasmid, i.e. 0, 100, 300 and 500 ng of pRcCMVp53, keeping the total amount of plasmid DNA at 1.5 µg with the empty vector pcDNA3. The luciferase activity that was determined in the absence of p53 was defined 100% in each case, and the other values were normalized accordingly.

**Figure 7: Requirement for p21 to negatively regulate gene expression by p53**

A. Levels of p53 and p21 protein in doxorubicin-treated cells

HCT116 cells, wild type or with targeted disruptions of the *p21/CDKN1A* or *p53* genes, were each treated with 350 nM doxorubicin. After 48 hours, the protein levels of p53 and p21 were determined by immunoblot analysis. Staining of actin served as a loading control. Above each lane, the presence (+) or absence (-) of doxorubicin is indicated, as well as the genotype of the cells under study, with disruptions (-) or wild type copies (+) of the *p53* and *p21/CDKN1A* genes. Note that p21 was not detected in cells lacking *p53*, despite the presence of the *p21/CDKN1A* gene in these cells (lanes 5 and 6). This was expected, since p53 appears as the principal inducer of *p21/CDKN1A*, and p21 levels are therefore below detectability in the absence of *p53*. 
B. Impact of p53 and p21 on RNA levels

HCT116 cells were treated as in A. After 48 hours, RNA was prepared, and the expression levels of the indicated genes were analyzed by RT-PCR as described in the legend to Fig. 1B.

**Figure 8: p21 as a negative regulator of gene expression**

A. Levels of p21 protein in transduced cells

H1299 cells were transduced with adenovirus vectors to express β-galactosidase, p53 or p21 as indicated. After 48 hours, the protein levels of p21 were determined by immunoblot analysis. Staining of actin served as a loading control.

B. Impact of p53 and p21 on RNA levels

H1299 cells were transduced as in A. After 48 hours, RNA was prepared, and the expression levels of the indicated genes were analyzed by RT-PCR as described in the legend to Fig. 1B.

**Figure 9: Influence of c-MYC on the mRNA levels of p21/CDKN1A, survivin and stathmin**

A. c-MYC as an antagonist of p53-mediated repression

H1299 cells were transduced with adenovirus vectors to express c-MYC and/or p53 in the indicated combinations (multiplicity of infection = 20). The mRNA levels of the indicated genes were determined by RT-PCR as described in the legend to Fig. 1B.

B. Failure of c-MYC to block p21-mediated repression

H1299 cell were transduced to express p21 and/or c-MYC, followed by RT-PCR analysis as in A.
Table 1: Genes that are negatively regulated by p53.

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Löhr et al., Figure 1:

A

$$\text{Ad-} \beta \text{-Gal} + \text{Ad-E1B-19kDa}$$

85 kDa $\rightarrow$ 120 kDa

Ad-p53wt $\begin{array}{c} - \text{Ad-} \beta \text{-Gal} + \text{Ad-E1B-19kDa} \\
\end{array}$

B

- total RNA
- $UbC$
- $Survivin$
- $STMN1$
- $PIG3$

Ad-p53wt $\begin{array}{c} - \text{Ad-} \beta \text{-Gal} + \text{Ad-E1B-19kDa} \\
\end{array}$

C

Survivin promoter

Luciferase activity (%)
Löhr et al., Figure 2

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- total RNA: 28S, 18S
- UbC: 322bp
- Survivin: 308bp
- STMN1: 319bp
- PIG3: 349bp

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Ad-p53wt: - + - +
Löhr et al., Figure 3

- β-Gal
- p53wt
- p53mt22/23

**total RNA**
- 28S
- 18S

**UbC**
- 322bp

**Survivin**
- 308bp

**STMN1**
- 319bp

**PIG3**
- 349bp
A

\[ \text{p73ΔN}\alpha \]
\[ \text{p73ΔN}\beta \]
\[ \text{p73TA}\beta \]
\[ \text{p53} \]

transactivation domain
DNA binding domain
oligomerization domain
steric alpha motif
post SAM domain

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28S
18S
322bp
349bp
308bp
319bp
**A**

Consensus sequence: \( \text{RRRCWGWYYY} \) \( N_{0-13} \) \( \text{RRRCWGWYYY} \)

**Survivin wild type:**
\( \text{GGGC} BG CgC \) TCC cGACATGCCC

**Survivin mutant:**
\( \text{GGGC} BG CgC \) TCC cGGGATGCCC

**p21 wild type:**
\( \text{GAACATGTCC} \) - cAACATGTTg

**p21 mutant:**
\( \text{GAAAATTTCC} \) - cAACATGTTg

\( R = A,G; \ W = A,T; \ Y = T,C \)

**B**

- + - + - + - + - + - + - + - -

Consensus sequence: \( \text{RRRCWGWYYY} \) \( N_{0-13} \) \( \text{RRRCWGWYYY} \)

**Survivin wild type:**
\( \text{GGGC} BG CgC \) TCC cGACATGCCC

**Survivin mutant:**
\( \text{GGGC} BG CgC \) TCC cGGGATGCCC

**p21 wild type:**
\( \text{GAACATGTCC} \) - cAACATGTTg

**p21 mutant:**
\( \text{GAAAATTTCC} \) - cAACATGTTg

\( R = A,G; \ W = A,T; \ Y = T,C \)
Löhr et al., Figure 6

A

wild type: CCGCCTAAGA GGGCGTGCGC TCCCGACATG CCCCGCGGCG CGCCATTAAC

mutant 1: CCGCCTAAGA GGGCGTGCGC TCCCGACATG CCCCGCGGCG CGCCATTAAC

mutant 2: CCGCCTAAGA GGGCGTGCGC TCCCGACATG CCCCGCGGCG CGCCATTAAC

mutant 3: CCGCCTAAGA GGGCGTGCGC TCCCGACATG CCCCGCGGCG CGCCATTAAC

mutant 4: CCGCCTAAGA GGGCGTGCGC TCCCGACATG CCCCGCGGCG CGCCATTAAC

B

Luciferase activity (%)

wildtype mutant 1 mutant 2 mutant 3 mutant 4

C

Luciferase activity (%)

wildtype mutant 1 mutant 2 mutant 3 mutant 4
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| doxorubicin | + | + | - | + | - | + |
| p21/CDKN1A | + | + | - | - | + | + |
| p53         | + | + | + | + | - | - |

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### B

| doxorubicin | - | + | - | - | + |
| p21/CDKN1A | + | + | - | - | + |
| p53         | + | + | + | - | - |

| total RNA  | 28S | 18S |
| UbC        | 322bp | 349bp |
| PIG3       | 308bp | 319bp |
| Survivin   | 403 bp | 355 bp |
| STMN1      | 490 bp | 551 bp |
| Cyclin B1  | 325 bp | 319 bp |
| TOP2α      | 325 bp | 325 bp |
| CHEK1      | 202 bp | 286 bp |
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25 kDa → p21
45 kDa → Actin

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p21/CDKN1A mediates negative regulation of transcription by p53
Kristina Löhr, Constanze Möritz, Ana Contente and Matthias Dobbelstein

J. Biol. Chem. published online May 13, 2003

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