

Activation of p53-p21^{waf1} Pathway in Response to Disruption of Cell-Matrix Interactions*

(Received for publication, June 5, 1997, and in revised form, August 8, 1997)

Ray-Chang Wu and Axel H. Schönthal‡

From the Department of Molecular Microbiology and Immunology, and K. Norris, Jr. Comprehensive Cancer Center, University of Southern California Medical School, HMR-405, Los Angeles, California 90033

The proliferation of most cells is strictly dependent on cell-matrix interactions, a phenomenon called anchorage dependence. Because tumor cells often are independent of this regulation, it is important to characterize the molecular pathways that control cellular proliferation after detachment of cells from their matrix. In this report, we investigated a possible role of p53 and one of its target genes, p21^{waf1/cip1}, as components of anchorage-dependent cell growth control. We found that p53 protein is rapidly activated upon the disruption of cellular attachment. This led to p21 transcriptional activation via two p53-binding sites in its promoter. Elevated p21 protein levels blocked transcription and activity of the cell cycle-regulator cyclin A, and cells became arrested in G₁ of the cell cycle. Under the same conditions, fibroblasts from p53 knock-out mice did not activate p21 and did not down-regulate cyclin A expression but rather induced another cell cycle inhibitor, p27. Thus, our results characterize a chain of events, starting from the activation of p53 and proceeding via p21 to cyclin A, that is activated in response to the loss of cellular adherence. This p53-regulated pathway may constitute one of a few redundant systems to ensure proper cell control in multicellular organisms.

The proliferation of normal cells, with the exception of some hematopoietic cells, is strictly dependent on cell-matrix interactions. Nonadherent mesenchymal cells fail to proliferate despite the presence of growth factors, a characteristic called anchorage dependence. In contrast, many transformed cells have lost their anchorage dependence and grow independently of cell-matrix interactions. This anchorage-independent phenotype in cell culture has been found to closely correlate with the ability of cells to form tumors in animals (1).

The proliferation of all cells, and their progression through the cell cycle, is regulated by the sequential activity of various cyclin-dependent kinases (cdks).¹ The enzymatic activity of cdks is dependent on posttranslational modifications, as well as on physical interactions with one of the cyclin proteins that are

the regulatory subunits of cdks (2, 3). The expression of cyclins is stimulated in response to growth factor stimulation of resting (G₀) cells and is required for cell cycle progression. In addition, there are two families of cdk inhibitors (CKIs) as follows: p21^{waf1/cip1}, p27^{kip1}, and p57^{kip2}, which bind to and inactivate most cyclin-cdk complexes, and the INK4s, which only inhibit complexes containing cdk4 and cdk6 (4, 5).

An important regulator of p21 transcription is the tumor suppressor protein p53 (6, 7). It is a transcription factor that binds to a specific DNA response element in the promoter of regulated genes, such as the muscle creatine kinase gene, the *GADD45* gene, and the *MDM-2* oncogene (8–10). In the promoter of the p21 gene, two such binding sites have been identified and shown to confer transcriptional activation by p53 (11). In addition, the p21 gene has binding sites for other transcription factors that may play a role in the regulation of p21 expression by p53-independent pathways (12–17).

A link between cyclin expression and anchorage-dependent cell cycle progression has recently been established. In mouse and human fibroblasts, the serum-stimulated induction of cyclin A and cyclin D1 expression was dependent on cell adhesion (18–20). In non-adherent cells, the expression of either mRNA and protein was blocked, and the cells were arrested in G₁ of the cell cycle. However, the forced ectopic expression of either cyclin A or cyclin D1 cDNA resulted in anchorage-independent cell division, suggesting that both cyclins might be targets of the adhesion-dependent signals that control cell proliferation. At least part of this control might be exerted at the transcriptional level. Although one report suggested the involvement of the E2F-binding site in the cyclin A promoter (21), another report indicated the presence of a novel CCAAT-binding protein that may mediate the adhesion-dependent transcriptional activation of cyclin A (22). In addition, another part of this control was exerted posttranscriptionally. It was shown that the kinase activity of cdk-cyclin E complexes was repressed after detachment of cells (18, 20).

However, there appear to be cell type-specific differences in the control of anchorage-dependent growth, not only between normal and tumor cells but also between cell lines that are strictly anchorage-dependent. For example, in anchorage-dependent NIH3T3 cells there is no cyclin D synthesis after serum stimulation of G₀-synchronized cells when the cells are detached (18). Consequently, the retinoblastoma protein, a substrate of cdk4-cyclin D complexes, cannot become phosphorylated. Hence, retinoblastoma protein stays in its hypophosphorylated, active form and prevents cells from entering S phase. In contrast, in NRK cells, which are also anchorage-dependent, cyclin D synthesis is stimulated under the same conditions, and retinoblastoma protein becomes phosphorylated (18). However, the cells are still arrested in the cell cycle because they are unable to induce cyclin A expression to a level sufficient for

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‡ Supported by a grant from the M. E. Early Medical Research Trust and by Grant CN-82601 from the American Cancer Society. To whom correspondence should be addressed: Dept. of Molecular Microbiology and Immunology, and K. Norris, Jr. Comprehensive Cancer Center, University of Southern California Medical School, 2011 Zonal Ave., HMR-405, Los Angeles, CA 90033. Tel.: 213-342-1730; Fax: 213-342-1721; E-mail: schontha@hsc.usc.edu.

¹ The abbreviations used are: cdks, cyclin-dependent kinases; CKIs, cdk inhibitors; HEMA, poly(2-hydroxyethyl methacrylate); EMSA, electrophoretic mobility shift assay; MEFs, mouse embryo fibroblasts; tk, thymidine kinase.

proliferation. Together, these data also emphasize a certain redundancy in adhesion-dependent cell cycle control (18–20).

In our report, we sought to investigate molecular events that took place early after the disruption of cell-matrix interactions, and we analyzed their possible connection to later processes, such as the regulation of cyclin expression. In our studies, we found that p53 protein is rapidly activated upon the disruption of cellular attachment of logarithmically growing cells. This led to the transcriptional activation of the p21 gene via the two p53-binding sites in its promoter region. Elevated p21 protein levels blocked cyclin A transcription and activity and subsequently led to proliferation arrest in G₁ of the cell cycle. In contrast, in cells lacking p53, p21 expression was not elevated, and cyclin A expression was not down-regulated; rather, in these cells another inhibitor of cyclin-dependent kinases, p27, was induced after the disruption of cell-matrix interactions. Thus, our results characterize a cell type-specific chain of events that starts from the activation of p53, proceeds via p21, and targets cyclin A. This p53-regulated pathway may constitute one of several partially redundant systems to ensure proper cell control in multicellular organisms.

EXPERIMENTAL PROCEDURES

Materials—HEMA (poly-HEMA; poly(2-hydroxyethyl methacrylate)) was obtained from Sigma and dissolved in ethanol at 10 mg/ml. Synthetic oligonucleotides for EMSAs were provided by the Core Facility of the K. Norris Jr. Comprehensive Cancer Center.

Cell Culture—C3 10T 1/2 mouse fibroblasts were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C in a 5% CO₂ atmosphere. Mouse embryo fibroblasts (MEFs) from p53 knock-out mice were kindly provided by Lawrence A. Donehower (Baylor College of Medicine, Houston, TX) and cultured as described above. For the disruption of cell-matrix interactions, monolayered cells were scraped off the culture dish and dispersed by pipetting. Then one-half was seeded back into a culture dish for re-attachment, and the other half was cultured in HEMA-coated plates which prevented the attachment of cells (23). Most experiments were repeated with agar-coated plates (1.4% agar in complete medium) in which attachment of cells was prevented as well.

Transient Transfections—Transient transfections were performed using the calcium-phosphate-DNA precipitation technique (24). The transfection mixture was added into the medium, and the cells were exposed to the precipitate for 12 h. Then the monolayer was washed twice with phosphate-buffered saline, and the cells were scraped off the plate. One-half was put into a fresh plate, the other half was put into a HEMA-coated plate. After another 24 h the cells were harvested, and cellular lysates were prepared. After determining the protein concentration by use of the bicinchoninic assay reagent (Pierce), the luciferase activity of each lysate was determined in a luminometer. In all transfections a plasmid encoding β -galactosidase (CMV- β -gal) was included to monitor transfection efficiencies. All transfections were repeated at least three times.

Plasmid Constructs—Luciferase reporter plasmids containing the human p21 promoter (11) as well as plasmid PG13 (containing 13 p53-response elements upstream of a polyoma basal promoter) (7) were obtained from Wafik El-Deiry (University of Pennsylvania, Philadelphia, PA). To generate plasmid p21-tk, a 1.1-kilobase pair fragment containing both p53-binding sites, was excised from the p21-wt plasmid (called WWP-luc in Ref. 17) with *Hind*III and *Acc*I. This fragment was inserted upstream of a minimal thymidine kinase (tk) promoter-luciferase reporter. The expression vector for p21 was generated by excising a 0.85-kilobase pair mouse p21 cDNA fragment from plasmid p21-9c (25) with *Eco*RI and inserting it downstream of a cytomegalovirus promoter in plasmid pCMV-blue (PharMingen, San Diego, CA). Construct cyclin A-luciferase (26) was obtained from Toshio Nikaido (Shinshu University, Matsumoto, Japan). PP2A-luciferase was generated by subcloning a 1200-base pair *Acc*I/*Hind*III fragment of the PP2A catalytic subunit α promoter (27) into pGL3-luc basic (Promega, Madison, WI).

RNA Analysis—Total RNA was isolated using the guanidinium thiocyanate method (28), followed by poly(A) extraction using oligo(dT) beads (29). Equal amounts of each RNA sample were separated on

formaldehyde/agarose gels and transferred onto nitrocellulose membranes. For hybridization, specific riboprobes were generated using T7 RNA polymerase according to manufacturer's instructions. The hybridization was carried out essentially as described (30). After hybridization, the membranes were washed twice at 80 °C in 0.2 \times SSPE and 0.5% SDS for 30 min and subsequently exposed to Kodak X-AR autoradiographic film. After exposure, the filters were stripped and rehybridized to a probe for β -actin to control for equal amounts of RNA loaded in each lane.

Western Blot Analysis—Cells were lysed in RIPA buffer as described (31). 20 μ g of each sample was separated by polyacrylamide gel electrophoresis and blotted onto nitrocellulose. After blocking with blotting (5% milk, 0.1% Tween 20, 10 mM Tris/HCl, pH 7.5, 150 mM NaCl) for 1 h, the membrane was exposed to the primary antibody diluted in blotting at 4 °C overnight. All antibodies against cell cycle-regulatory proteins were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and diluted according to the manufacturer's instructions. The secondary antibodies were coupled to horseradish peroxidase and were detected by chemiluminescence using the SuperSignal™ Substrate (Pierce).

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts from 10⁷ cells per point were prepared exactly as described previously (32). The protein concentration was measured using the bicinchoninic acid assay (Pierce) with bovine serum albumin as the standard. The binding reaction was performed by incubating 12 μ g of nuclear extract with 0.5 μ g of poly(dI-dC) and ³²P-labeled oligonucleotides in binding buffer (5 mM HEPES, pH 7.8, 5 mM MgCl₂, 50 mM KCl, 0.5 mM dithiothreitol, 10% glycerol) in a final volume of 20 μ l. The sequences for the double-stranded, *in vitro* synthesized oligonucleotides were as follows: p53-binding site at position -2800 of the mouse p21 promoter, 5'-GGAAC-ATGTCTTGACATGTC-3'; inactivated binding site, 5'-GGAATATAT-CTTGAATTCCTTC-3' (nucleotides that deviate from the wild type binding site are underlined). These double-stranded oligonucleotides were end-labeled with [γ -³²P]ATP and T4 polynucleotide kinase, purified over a column, and used at 4 \times 10⁴ cpm per reaction. To produce supershifts, the nuclear extracts were incubated with 100 ng of mouse monoclonal antibody against p53 (PAb 421, Calbiochem) for 30 min prior to the addition of oligonucleotides (33). For competition purposes, 100 ng (160-fold molar excess) of unlabeled wild type or mutant p53 oligonucleotides were included in the binding reaction. All binding reactions were incubated for 30 min at room temperature and separated on a 5% acrylamide gel.

RESULTS

Several previous studies that analyzed the molecular mechanisms of anchorage dependence were performed by using G₀-synchronized cells (18–20). The studies showed that these cells, after detachment, were unable to induce expression of cyclin A and cyclin D1 in response to stimulation with serum growth factors and were not able to progress through the cell cycle. In our study presented here, we used cells that were growing logarithmically, *i.e.* that were distributed throughout the cell cycle at the onset of detachment. In contrast to G₀-synchronized cells where the expression of cyclins is shut down and cdk activity is marginal, logarithmically growing cell cultures exhibit high levels of cyclin expression and cdk activity. Our goal was to determine how this highly active cell cycle machinery was controlled after the loss of cellular adherence.

10T 1/2 murine fibroblasts were either cultured in plastic tissue culture dishes (adherent) or on top of agar or HEMA-coated dishes (non-adherent) in the continuous presence of serum growth factors. Under non-adherent conditions, the cells became growth-arrested and accumulated in G₁ of the cell cycle (not shown). At the same time, the kinase activity associated with cyclin A-containing cdks was down-regulated rapidly (Fig. 1).

To determine the mechanisms of inhibition of cdk activity, we next analyzed the protein levels of various cdks and cyclins (Fig. 2). Cyclin A protein was down-regulated and became undetectable at 36 h after the onset of detachment. Cyclin D1 was down-regulated as well but was still detectable after 48 h. There was also some decrease in cdc2 protein levels, and no

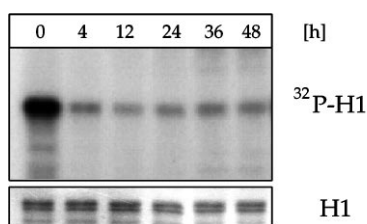


FIG. 1. Anchorage-dependent cyclin A-cdk activity. Cells were detached and cultured on HEMA-coated plates to prevent adherence. At the indicated time points, the cells were harvested, and cdk complexes containing cyclin A were immunoprecipitated with anti-cyclin A antibodies. The *in vitro* kinase reaction was performed with histone H1 protein as a substrate and separated on an acrylamide gel as described (30). The *top panel* is an autoradiograph showing ^{32}P -phosphorylated histone H1 (^{32}P -H1). The *bottom panel* shows the same gel stained with Coomassie Blue as a control for the same amounts of histone H1 used in each kinase reaction. This experiment was repeated several times with similar results.

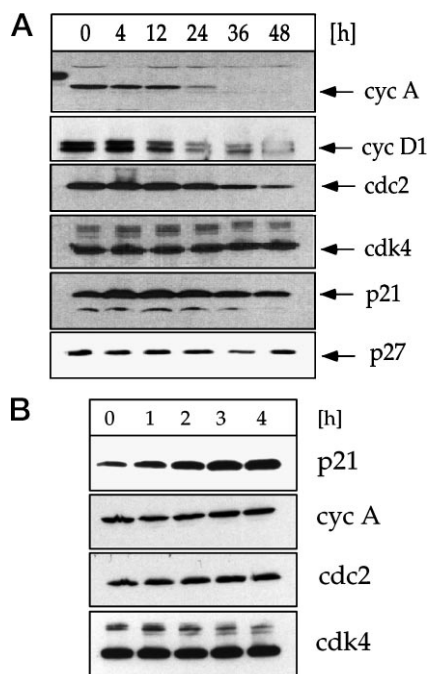


FIG. 2. Amounts of cell cycle-regulatory proteins in non-attached cells. Cells were detached and cultured on HEMA-coated plates to prevent adherence. At various time points, the cells were harvested, and the amount of the indicated proteins was determined by Western blot analysis with specific antibodies. Shown are late (A) and early time points (B). *cyc A*, cyclin A; *cyc D1*, cyclin D1. For experimental details, see "Experimental Procedures."

decrease in cdk4 protein. Importantly, however, none of these proteins exhibited a decrease within the first 4 h (Fig. 2B), which is the period when cyclin A-containing complexes lost most of their kinase activity. This finding indicated the effects of two regulatory mechanisms: the first one at the post-translational level which controls the enzymatic activity of the kinase (within the first few hours) and the second at the level of expression which controls the synthesis of the various kinase subunits (a later event).

Because $p21^{uaf1/cip1}$ and $p27^{kip1}$ are known posttranslational regulators of cdk activity, we analyzed their expression levels during the non-attached culture of cells (Fig. 2A). Whereas the amount of p27 protein did not increase during the course of the experiment, p21 protein levels increased 3-fold after detachment. Because this increase was already maximal at 4 h, we analyzed its kinetics of induction at earlier time points as well. We found that p21 expression was induced very

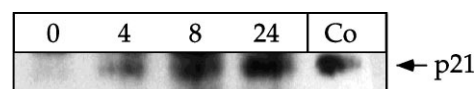


FIG. 3. Amount of p21 protein in cyclin A-cdk complexes. Cells were detached and cultured on HEMA-coated plates for the indicated times. Total cellular lysates were immunoprecipitated with anti-cyclin A antibodies. The immunocomplexes were collected with protein A-agarose and separated by denaturing polyacrylamide gel electrophoresis. After transfer to nitrocellulose membrane, p21 protein was detected by Western blot analysis. *Co*, control lane containing total cellular lysate (to confirm location of p21 protein). This experiment was repeated twice with similar results.

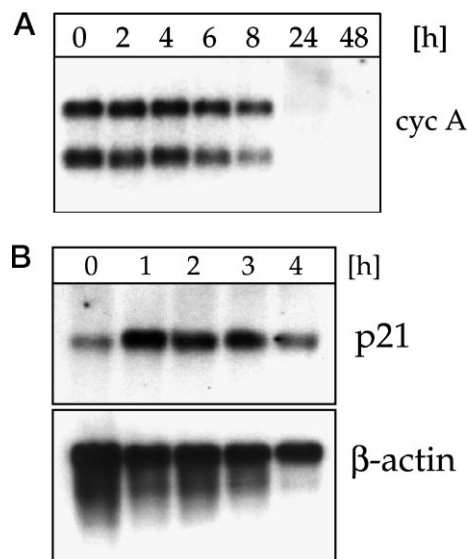


FIG. 4. Northern blot analysis of detached cells. Cells were detached and cultured on HEMA-coated plates to prevent adherence. At the indicated time points, the cells were harvested, and the levels of cyclin A (*cycA*) (A) and p21 mRNA were determined by Northern blot analysis (B). As a control, the filters were also hybridized with a probe for β -actin mRNA (not shown for cyclin A filter). For experimental details, see "Experimental Procedures." A, both bands are specific for cyclin A mRNA and are usually observed in murine cells.

early after detachment. As shown in Fig. 2B, the increase in p21 protein could be detected already after 1 h and reached its maximum at 3–4 h. At these time points, there was no change in the levels of cyclin A, cyclin D1, cdc2, and cdk4 proteins (Fig. 2B). Because cdk4 containing cyclin A are known targets for p21, our results suggest that the posttranslational inhibition of cdk activity after detachment of cells may be due to the increased levels of the cdk inhibitor p21.

To test this directly, we investigated the amount of p21 protein in cyclin A-containing protein complexes. For this purpose, cyclin A was immunoprecipitated, and the collected complexes were analyzed for their p21 content by Western blotting. As shown in Fig. 3, the amount of p21 protein associated with these complexes was greatly increased after the loss of cellular adherence. This finding directly implicated p21 as a negative posttranslational regulator of cdk activity in response to cell-matrix disruption.

To determine further whether elevated levels of p21 protein may also be involved in the observed down-regulation of cyclin A expression, we investigated the mechanisms of cyclin A down-regulation. As shown in Fig. 4A, the mRNA levels of cyclin A decline after cellular detachment and are undetectable at 24 h thereafter. This down-regulation appeared to start around 6 h, which is well after the induction of p21 protein (Fig. 2) and p21 mRNA (Fig. 4B). This finding raised the possibility that elevated levels of p21 may result in decreased expression of cyclin A. To test this more directly, we co-transfected an

expression vector containing p21 cDNA together with a luciferase reporter construct under the control of the cyclin A promoter. This co-transfection was performed in adherent cells to avoid the induction of the endogenous p21 gene (which occurs after detachment of cells). As shown in Fig. 5, elevated levels of p21 caused a decrease in cyclin A promoter activity, indicating that p21 protein is able to inhibit, directly or indirectly, transcription from the cyclin A promoter. Similarly, after detachment of cells, *i.e.* under conditions where the amount of endogenous p21 protein is increased, the activity of a transfected cyclin A promoter is reduced as well (not shown). As a control, we also transfected the promoter of a different gene, protein phosphatase type 2A. In this case, no down-regulation was observed neither during detachment (not shown) nor with co-transfected p21 cDNA (Fig. 5), indicating that the observed inhibitory effects on the cyclin A promoter were specific. Rather, there was a weak (1.6-fold) induction of the PP2A promoter. As PP2A has been characterized as a negative regulator of cell growth (34), this effect may warrant some further investigation in the future.

The results above indicated the involvement of p21 protein in the transcriptional and post-translational inhibition of cyclin A expression and activity during cellular detachment. Because this control seemed to be exerted through elevated levels of

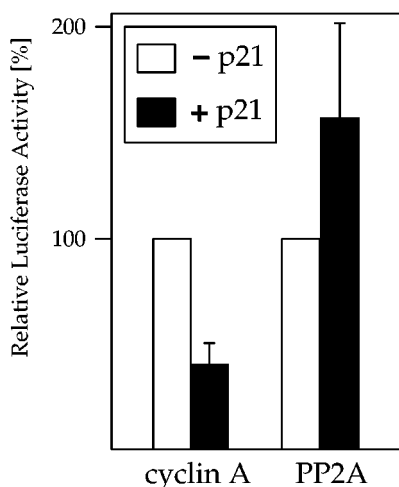
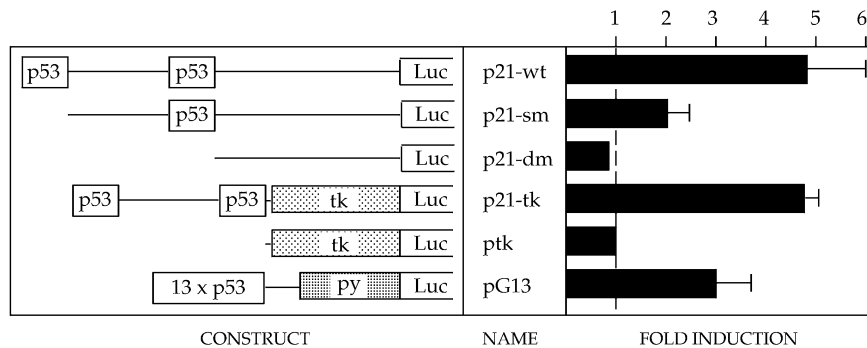


FIG. 5. Cyclin A promoter activity in the presence of transfected p21 cDNA. Cells were grown attached to cell culture dishes. They were transiently co-transfected with 4 μ g of cyclin A-luciferase reporter construct (cyclin A) together with 2 μ g of either an expression vector for p21 (+p21, black bars) or the same expression vector without the p21 cDNA insert (-p21, white bars). As a control, p21 cDNA was also co-transfected with a luciferase reporter plasmid under the regulation of the promoter for serine/threonine protein phosphatase type 2A (PP2A). 24 h after transfection, the cells were harvested, and luciferase activity was determined. Shown is the average of 3–5 independent experiments. The luciferase activity from cells without co-transfected p21 cDNA was arbitrarily set at 100%. Error bars reflect standard deviation.

FIG. 6. Activation of the p21 promoter via p53-binding sites. Cells were transiently transfected with the indicated constructs. After transfection, the cells were scraped off the culture dish and divided in two. One-half was seeded back into a culture dish, and the other half was cultured on top of HEMA-coated plates. 24 h later all cells were harvested and analyzed for luciferase activity. Shown is the ratio of luciferase activity between cells that were kept in suspension and cells that had re-attached to the tissue culture plate. Each bar represents the average of >3 independent experiments.



p21, and because the tumor suppressor protein p53 had been shown to be a major regulator of p21 expression, we investigated whether the increased expression of p21 during detachment was controlled by p53.

Two p53-binding sites have been identified in the p21 promoter and shown to bind p53 protein (11). One is located at -2225 and the other at -1330 with respect to the start of transcription of the gene. We used p21 promoter deletion mutants containing either both, only one, or none of the p53-binding sites, fused to the luciferase reporter gene (Fig. 6). After transfection of these constructs, the cells were split in half and cultured either under adherent or non-adherent conditions. As shown in Fig. 6, the p21 promoter containing both p53-binding sites (construct p21-wt) was induced nearly 5-fold after detachment of cells, as compared with adherent cells. When only one p53-binding site was present (construct p21-sm) this induction was reduced to 2-fold. The construct where both p53-binding sites had been deleted (p21-dm) was not induced at all.

To confirm the importance of the p53-binding sites further, we transferred the fragment of the p21 promoter, which contained the two binding sites, to a heterologous promoter, the thymidine kinase (tk) promoter. The tk promoter alone, fused to the luciferase gene (ptk), was not induced after detachment of cells. However, after insertion of the two p53-binding sites (p21-tk), this construct was induced nearly 5-fold (Fig. 6). In addition, a construct containing 13 repeats of a consensus p53-binding site (pG13) was induced 3-fold after cellular detachment. Thus, these results demonstrate that the p53-binding site is necessary and sufficient for the induction of p21 during non-adherent culture conditions.

The activation of p53 was confirmed by electrophoretic mobility shift assays (EMSAs). Extracts from adherent or non-adherent cells were incubated with an oligonucleotide representing the distal p53-binding site of the p21 promoter. This sequence has been shown before to bind p53 protein (11). As can be seen in Fig. 7, there was four times more binding activity in cells that were detached, indicating elevated p53 activity. A mutated oligonucleotide with several point mutations did not compete for specific binding and did not exhibit any increased binding in detached cells. Together with the observed transcriptional activation via the p53-binding sites, these experiments demonstrate activation of p53 after detachment of cells.

Our data so far indicated that disruption of cell-matrix interactions activated a chain of events that involve p53, followed by the transcriptional activation of p21, which is followed by the transcriptional and posttranslational inhibition of cyclin A. We therefore investigated next whether or not these events would take place in cells that lack functional p53 protein. We used mouse embryo fibroblasts (MEFs) from p53 knock-out mice (p53^{-/-}) and determined the levels of cyclin A and p21 protein after disruption of cell-matrix interactions. As shown in

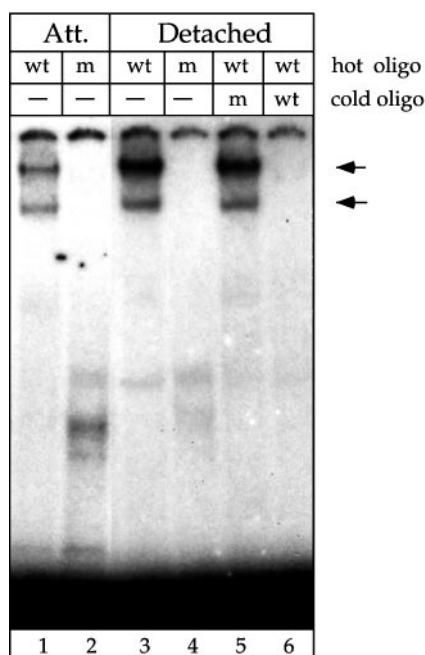


FIG. 7. DNA binding activity of p53 in attached versus non-attached cells. Nuclear lysates from cells that were either grown attached (*Att.*) or non-attached for 2 h were prepared and subjected to electrophoretic mobility shift assays (EMSA) with an oligonucleotide representing the distal p53-binding site in the p21 promoter (wild type, *wt*). As a negative control, we also used a mutated version of this oligonucleotide (*m*) that was unable to bind p53 protein. In addition to nuclear extract, the individual reactions contained the following: *lanes 1 and 3*, 32 P-labeled wild type oligonucleotide; *lanes 2 and 4*, 32 P-labeled mutant oligonucleotide; *lane 5*, 32 P-labeled wild type oligonucleotide plus 160-fold molar excess of non-radioactive mutant oligonucleotide; *lane 6*, 32 P-labeled wild type oligonucleotide plus 160-fold molar excess of non-radioactive wild type oligonucleotide. *Hot oligo* in the figure refers to 32 P-labeled oligonucleotide and *cold oligo* to non-radioactive oligonucleotide. To enhance the specific DNA binding activity of p53 (10, 42), anti-p53 antibodies were included in each reaction. The *two arrows* on the *right* point to shifted bands that are specific for p53 binding. This experiment was repeated with essentially the same result.

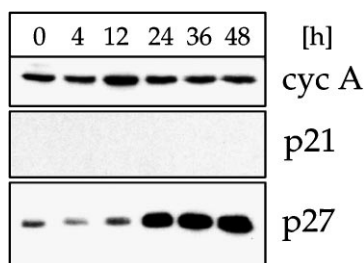


FIG. 8. Amount of cell cycle-regulatory proteins in p53^{-/-} cells after detachment. Mouse embryo fibroblasts (MEFs) from p53 knock-out mice were detached and cultured on HEMA-coated plates for the indicated times. Then cells were harvested, and the amount of the indicated proteins was determined by Western blot analysis with specific antibodies. *cyc A*, cyclin A. Shown is one representative experiment of several with similar results. The procedure and exposure time for these Western blots are similar to the ones shown in Fig. 2.

Fig. 8, the amount of cyclin A protein is not reduced after cellular detachment, which is in contrast to the p53-positive mouse fibroblasts used in Fig. 2A. Furthermore, the level of p21 protein was below detection limits in these MEF (p53^{-/-}) cells (Fig. 8). As p21 needs to be present in fairly high amounts (approximately 2:1 molar ratio of p21 to cdk) to act as a cdk inhibitor (35, 36), it is unlikely that the extremely low amounts of p21 protein in the MEF (p53^{-/-}) cells are sufficient to act as cdk inhibitor. Interestingly, the amount of another CKI,

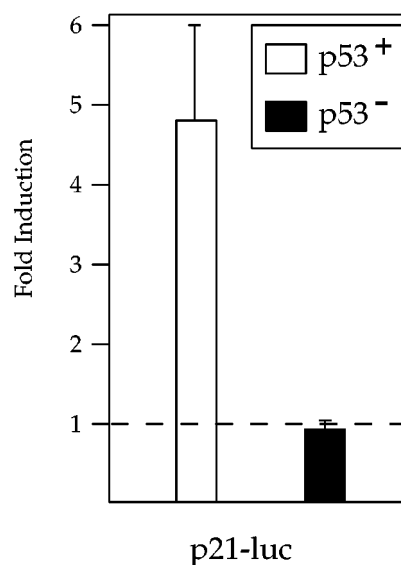


FIG. 9. p21 promoter activity in mouse fibroblasts with or without p53. 10T 1/2 cells (p53⁺) or MEFs from p53 knock-out mice (p53⁻) were grown attached to cell culture dishes. They were transiently transfected with 5 μ g of p21-luc, which contains the p21 promoter upstream of the luciferase reporter gene. After transfection, the cells were detached, and one-half was seeded back onto a regular culture dish (for adherence), and one-half was cultured in HEMA-coated plates (detached). 12 h later, the cells were harvested and luciferase activity was determined. Shown is the average ratio of luciferase activity in detached versus adherent cells from three independent experiments. To control for the transfection efficiency, CMV- β -gal was included in one experiment.

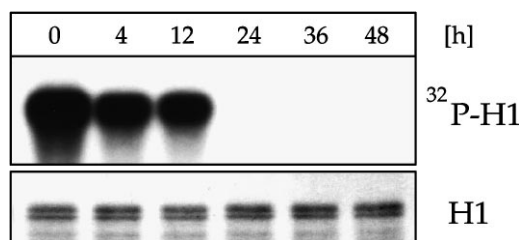


FIG. 10. Anchorage-dependent cyclin A-cdk activity in p53 negative cells. MEFs from p53 knock-out mice were detached and cultured on HEMA-coated plates to prevent adherence. At the indicated time points, the cells were harvested, and cdk complexes containing cyclin A were immunoprecipitated with anti-cyclin A antibodies. The *in vitro* kinase reaction was performed with histone H1 protein as a substrate and separated on an acrylamide gel as described (30). The *top panel* is an autoradiograph showing 32 P-phosphorylated histone H1 (32 P-H1). The *bottom panel* shows the same gel stained with Coomassie Blue as a control for the same amounts of histone H1 used in each kinase reaction.

p27^{kip1}, was strongly elevated at around 24 h after detachment of the MEF (p53^{-/-}) cells, which is in contrast to the p53-positive mouse fibroblasts, where no change is observed (Fig. 2A).

To confirm the observed lack of p21 activation in MEF (p53^{-/-}) cells, we transfected the p21 promoter, fused to the luciferase reporter gene, into these cells and determined its activity after disruption of cell-matrix interactions. As shown in Fig. 9, there is no activation of the p21 promoter after cellular detachment, which is in contrast to the 5-fold activation of the same construct in the p53-positive cells. Together with the above described lack of p21 protein, these results demonstrate that p21 is not activated in response to disruption of cell matrix-interactions in MEF (p53^{-/-}) cells.

Finally, we determined the kinase activity of cdk complexes containing cyclin A in MEF (p53^{-/-}) cells after cell-matrix

disruption. Cyclin A-containing complexes from detached cells were immunoprecipitated and subjected to *in vitro* kinase reactions. As shown in Fig. 10, during the first 12 h after detachment, there is only a 2-fold reduction in cyclin A-associated kinase activity, whereas thereafter a much stronger reduction of kinase activity (>25-fold) occurs. This is in contrast to the p53-positive cells where all of the down-regulation of kinase activity occurs within the first 4 h (Fig. 1). Thus, the down-regulation of cyclin A-associated kinase activity in the p53-positive cells correlates with increased p21 expression, whereas the major down-regulation of cyclin A-associated kinase activity in MEF (p53^{-/-}) cells correlates with increased expression of p27. Overall, the chain of events that we observe in p53-positive mouse fibroblasts, namely the rapidly increased levels of p21, followed by the prompt inhibition of cyclin A expression and activity, is not observed in the cells lacking p53. Thus, this further indicates a critical role of p53 in these processes.

DISCUSSION

The proliferation of most normal cells is strictly dependent on cell-matrix interactions, a phenomenon called anchorage dependence. In light of the close correlation of the anchorage-independent phenotype of tumor cells and their tumorigenic potential (1), it is important to fully understand the control mechanisms that determine the cell growth arrest of logarithmically growing cells after the disruption of cell-matrix interactions. In this paper we have analyzed some of the molecular events that take place after the detachment of 10T 1/2 fibroblasts, an anchorage-dependent murine cell line, and compared them to murine fibroblasts from p53 knock-out mice, which are also anchorage-dependent. Our experiments differ from earlier studies by others (18–20) in that we analyzed logarithmically growing cells instead of G₀-synchronized cell cultures. We reasoned that proliferating cells, which harbor growth factor-activated signal transduction pathways and a highly operative cell cycle machinery, might use and reveal additional layers of control to achieve growth inhibition in response to cellular detachment.

In this report, we demonstrate that the expression of the cdk inhibitor p21^{waf1/cip1} was rapidly activated after detachment of cells. This activation was due to the increased DNA binding and transcriptional activity of the tumor suppressor protein p53. Elevated p21 protein levels led to the posttranslational and transcriptional inhibition of cyclin A, an essential cell cycle regulator. Subsequently, cells were arrested in G₁ of the cell cycle. The involvement of p53 in these processes was established at several levels as follows: (i) after the disruption of cell-matrix interactions, the DNA binding activity of p53 was increased 3–4-fold (Fig. 7); (ii) the activation of the p21 promoter after detachment occurred through its established p53-binding sites (Fig. 6); (iii) transfer of the p53-binding sites to a heterologous promoter also transferred the inducibility after cellular detachment (Fig. 6); (iv) in cells lacking p53, there was no induction of the p21 promoter (Fig. 9) and no detectable increase in p21 protein levels (Fig. 8) after detachment of cells. Together, these results clearly indicate the activation of the p53-p21 pathway in response to the disruption of cell-matrix interactions.

The observed activation of the p53-p21 pathway could be linked to the regulation of cyclin A expression and activity. Cyclin A has been shown before to be involved in anchorage-dependent growth control (19, 21, 22). Activation of p53 and p21, which occurred within the first few hours after detachment of cells, had two consequences: (i) a rapid posttranslational inhibition of cyclin A-associated kinase activity (within the first four hours, Fig. 1), which was likely due to the increased presence of p21 protein in cyclin A-cdk complexes

(Fig. 4); and (ii) a transcriptional inhibition of the cyclin A promoter (Figs. 4A and 5), which was a later event probably due to the inhibition of cdk activity (see below). In cells lacking p53, the expression of cyclin A was not inhibited (Fig. 8), and the major down-regulation of cyclin A-associated kinase activity only occurred at much later time points (between 12 and 24 h, Fig. 10). This posttranslational inhibition in p53-negative cells correlated with the increased expression of another CKI, namely p27^{kip1} (Fig. 8), and thus indicated that in these cells cdk activity in response to cellular detachment may be regulated by a different pathway involving p27. Taken together, our data indicate that in the p53-positive cells the activation of the p53-p21 pathway negatively impinges on the transcriptional and posttranslational activity of cyclin A. In a different system, a similar negative effect of p21 on cyclin A expression has recently been described in cells exposed to genotoxic stress (37).

The mechanism of down-regulation of the cyclin A gene by p21 is probably an indirect effect that is mediated via the transcription factor E2F (37). Other results have indicated that the activity of G₁ cyclin-cdk complexes, such as cyclin D-cdk4 and cyclin E-cdk2, is necessary for cyclin A expression (18, 21, 38). These kinases phosphorylate the retinoblastoma protein and release transcription factor E2F, which subsequently binds to and activates the cyclin A promoter (21). Furthermore, an additional level of regulation has been suggested by the finding that p21 is able to disrupt the interaction of complexes consisting of cdk2, E2F, and retinoblastoma family proteins (39, 40). In addition, another report indicated that adhesion-dependent cyclin A transcription is regulated by a novel CCAAT-binding protein, CBP/cycA, which itself is sequestered by a retinoblastoma-like protein (22). In view of these earlier findings, it is likely that after detachment the down-regulation of cyclin A expression by p21 might be controlled via the p21-mediated inhibition of cdk activity. This is supported further by the kinetics of reduced cyclin A mRNA levels; this reduction begins approximately 6 h after the disruption of cell-matrix interactions, which is well after the onset of diminished cdk activity (compare Figs. 1 and 4A).

In addition to its (indirect) effect on cyclin A gene expression, p21 also exerted its known (direct) function as a posttranslational inhibitor of cdk activity. The kinase activity of cyclin A-cdk complexes was strongly inhibited in response to the disruption of cell-matrix interactions (Fig. 1). This result is in accordance with findings by others (19, 20) who also described a lack of cyclin A activity in detached cells. However, the basis for this lack of activity is quite different between these studies. In the two other studies, cyclin A-associated kinase activity is lacking because the (G₀-synchronized) detached cells do not synthesize cyclin A protein in response to serum stimulation, that is there is no cyclin A protein available, and the inhibitory effect most likely reflects a transcriptional block of cyclin A expression. In contrast, in our study, cyclin A protein is present at high levels (Fig. 2) but is inactivated because of the posttranslational association with p21 protein (Fig. 3); the transcriptional inhibition of cyclin A expression only occurs at later time points and reflects an indirect effect of p21, as discussed further above. Thus, our data reveal two consecutive actions of anchorage-dependent growth control: first, the activation of the p53-p21 pathway leads to the rapid posttranslational inhibition of cdk activity; and second, the subsequent transcriptional shut down of cyclin A (and probably other genes) aids to secure long term growth arrest of detached cells.

In addition to p21, another CKI, p27^{kip1}, has been shown earlier to be activated in response to the disruption of cell-matrix interactions (18, 20, 21) and to be able to regulate cyclin

A gene expression (38). Our findings indicate that the induction of the respective CKI in response to cellular detachment is cell type-specific and may depend on the genotypic background of the cell. For instance, in our mouse fibroblast cell line that contains functional p53, we observe induction of p21 but not p27 (Fig. 2), whereas in mouse fibroblasts lacking p53, p27, but not p21, is induced (Fig. 8). Furthermore, the induction of p21 is rapid (within the first few hours after detachment) and correlates with the rapid inhibition of cyclin A-associated kinase activity (Fig. 1), whereas induction of p27 occurs much later and correlates with a much slower inhibition of kinase activity (Fig. 10). Clearly, in the p53-positive cells that we used, p21 appears to be a major trigger of growth arrest after cellular detachment, because (i) p21 induction occurs very early after detachment (Figs. 2B and 4B); (ii) cdk activity is inhibited rapidly (Fig. 1); and (iii) increased amounts of p21 protein can be detected in cyclin-cdk complexes (Fig. 3).

Our observation of the rapid down-regulation of cyclin A-associated kinase activity, in the continued presence of high levels of cyclin A protein, has implications for cause and effect relationships. It had been suggested that the absence of cyclin A in detached cells may be a consequence of cell cycle arrest, possibly because of the lack of active cyclin D- and cyclin E-containing cdk complexes, which would leave the retinoblastoma protein hypophosphorylated and E2F inactive (20). However, at least in our experimental system, this does not appear to be the case, because cell cycle arrest is not complete before approximately 20 h after detachment (not shown), whereas the decrease in cyclin A mRNA levels, and cyclin A-associated kinase activity, appears much earlier. In support of this, Zhu *et al.* (18) suggested that during the regulation of anchorage-dependent growth, cyclin A may provide a separate, but redundant, layer of control. For example, the control of anchorage-dependent growth in NRK cells appears to be mediated mainly via the regulation of cyclin A, as the forced expression of cyclin A renders the cells at least partially anchorage-independent (19).

Our results support the idea of the presence of redundant layers of anchorage-dependent growth control. As shown in this report and others (18–22), this regulation appears to involve several players, among them the CKI's p21 and p27, which act on cdk complexes containing cyclin A, cyclin D, or cyclin E. In addition, there is significant control at the transcriptional level, which we show here may involve the p53-p21 pathway.

Because p21 emerged as an important component of the anchorage-dependent growth control (this study and Ref. 20), we further investigated the signal transduction pathways that might impinge on p21 expression in response to the disruption of cell-matrix interactions. Expression of the p21 gene has been shown before to be responsive to p53-dependent, as well as p53-independent, pathways (6, 7, 9, 10). In this report, we demonstrated that during detachment, p53 became activated and stimulated expression of the p21 gene via the cognate p53-binding sites. Interestingly, however, we were not able to detect increased levels of total p53 protein in response to cellular detachment (not shown). This is different from the cellular response to genotoxic agents, which initiate a rapid and substantial increase in p53 protein levels (8) and suggest a different mechanism of p53 activation. Since p53 activity is also dependent on its phosphorylation status, it is conceivable that posttranslational modifications may be responsible for the activation of its transcriptional function in response to cellular detachment, although this needs to be established. Furthermore, it should be noted that the apoptotic function of p53 was not activated by cell-matrix disruption, as the detached cells remained viable for at least 48 h.

Taken together, our findings add yet another role for p53 to its expanding repertoire. As pointed out above, cell growth arrest in response to cellular detachment is likely regulated by several layers of control. Therefore, the above described pathway, which comes from the activation of p53 and proceeds via p21 to the regulation of cyclin A, may be one of a few such pathways that mediate cell growth arrest. This idea is further supported by the finding of others that p53-negative mouse embryonic fibroblasts (p53^{-/-} MEFs) still undergo cell cycle arrest in response to detachment (41). However, this growth arrest of p53^{-/-} MEFs, but not of p53^{+/+} MEFs, can be overcome by transformation with oncogenes (41). Thus, the lack of p53-mediated control enables these transformed cells to grow anchorage-independently. Taken together with other published data (18–20) and our own results, this seems to indicate that the presumed redundancy of anchorage-dependent growth control may consist of different layers that do not completely overlap or that may be used differentially depending on other factors, such as a cell's genetic background.

In any case, there appear to be circumstances where p53-regulated pathways appear to “dominate” the control of anchorage-dependent growth. Our results suggest a molecular basis for this control by demonstrating activation of p53, followed by the induction of p21 and the inhibition of cyclin A. As anchorage-independent growth correlates closely with the tumorigenic and metastatic potential of cells (1), our results further add to the notion that inactivation of p53 generates a higher probability for cells to form metastasis.

Acknowledgments—We are grateful to Wafik El-Deiry and Yi-Xin Zeng for providing several p21-luciferase constructs. We thank Lawrence A. Donehower for cells from p53 knock-out mice, and Konrad Huppi, Brian Hemmings, and Toshio Nakaïdo for kindly providing plasmids. The assistance of Zora Baharians and Silvina Campos is acknowledged.

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