

Reactivation of Mutant p53 by a One-Hybrid Adaptor Protein¹

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Abstract

The most frequent genetic alteration in cancer is a mutation of p53. In most cases, this leads to a sharp increase of the p53 protein levels but abolishes p53's function as an activator of transcription. To correct this defect, wild-type p53 is being reintroduced into tumor cells through gene therapy vectors, thereby inducing cell death. However, this effect is not necessarily specific for tumor cells. Furthermore, mutant p53 in tumor cells *trans*-dominantly impairs the function of wild-type p53. As an approach to overcome these obstacles, we have developed an adaptor protein that reactivates mutant p53 rather than stimulating transcription on its own. The DNA binding and tetramerizing portions of the p53-homologue p73 were fused to the oligomerization domain of p53. This chimera binds to the DNA of p53-responsive promoters through the p73-derived portions, and it binds to mutant p53 by the p53-derived oligomerization domain. Through this one-hybrid system, mutant p53 is re-enabled to activate transcription. When the adaptor was expressed in tumor cells that contain mutant p53, expression of p53-responsive genes was activated, and growth was inhibited. No such effects were observed in cells that contain wild-type p53 or no p53 at all. When the adaptor was expressed through an adenovirus vector, tumor cells containing mutant p53 were specifically induced to undergo apoptosis. This strategy can turn mutant p53 into an inhibitor of tumor cell growth and might enable gene therapy to eliminate cancer cells with specificity.

Introduction

p53 is mutated in ~50% of human malignancies. In most cases, these mutations result in the expression and accumulation of a stabilized protein with single amino acid substitutions. Wild-type p53 activates transcription through direct interaction with promoter DNA. The COOH-terminal portion of p53 (amino acids 294–393) oligomerizes to form a tetramer of p53 molecules. The central domains (amino acids 92–293) then interact cooperatively with a cognate DNA element. The NH₂-terminal portions (amino acids 1–61) bind to components of the basal transcription machinery and activate mRNA synthesis. The vast majority of p53 mutations in tumors are found within the central domain, impairing the ability of the protein to bind DNA, but retaining the functions of the transactivation and oligomerization domains (1).

Many attempts have been made and are still going on to correct the defects caused by p53 mutations using gene therapy. Wild-type p53 is being reintroduced into tumor cells using appropriate vectors, thereby inducing cell death (2, 3). However, this effect is not necessarily specific for tumor cells. Furthermore, mutant p53 in tumor cells

trans-dominantly impairs the function of reintroduced wild-type p53 (4).

A splice variant of the p53-homologue p73, termed p73TAβ (5), binds to the DNA and activates transcription of several p53-responsive promoters even more efficiently than p53 itself (Ref. 6 and our unpublished observations). As with p53, p73TAβ efficiently blocks cell growth and induces apoptosis (7). We took advantage of these similarities between p53 and p73 to create a chimera that might be usable to reactivate mutant p53.

Materials and Methods

Cell Culture, Infection, and Transfections. H1299, MDA-MB-468, C33A, and MRC5 cells were maintained in DMEM (Life Technologies, Inc.), A431 cells in RPMI. Fetal bovine serum (10%) was added to all media. For infection, 2×10^5 cells in a 3.5-cm dish were washed with PBS (136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, and 1.5 mM KH₂PO₄) and then overlaid with the indicated amount of virus in 500 μl of DMEM without serum. Transfections were done using Lipofectamine 2000 (Life Technologies, Inc.), followed by luciferase assays (Promega) 24 h after transfection or by selection with G418 (800 μg/ml; Sigma) 3 days after transfection.

Plasmids. To create an expression plasmid for the p53/p73 chimera, the transactivation domain of p73TAβ was deleted and replaced by a *Bam*HI-*Not*I linker within a pcDNA3 (Invitrogen)-based expression plasmid (generous gift of D. Caput), using the QuikChange protocol (Stratagene) and the mutagenesis primer CCGATCCGCCACCATGGCGGCCGCCGACCTTCCCCAGTC and its reverse complement to obtain pcDNA3p73βBN. Subsequently, the coding region for the oligomerization domain (residues 313–364) of human p53 was PCR-amplified with the primers GGCGGATCCGCCACCATGAGCTCCTCTCCCCAGCCAAAG and GACGGCGCGGCCGCCACCAGCCCTGCTCCCCCTGGCTC and cloned into pcDNA3p73βBN using *Bam*HI and *Not*I to obtain pcDNA3adaptor. For stable transfections, the insert was excised from pcDNA3adaptor with *Bam*HI and *Xba*I, filled in, and cloned into the vector pIRESneo (Clontech) that was cut with *Eco*RV, thereby obtaining pIRESneoAdaptor. The oligomerization domain of p53 within the adaptor was mutated using the mutagenesis primer GGAGAATATTTACCGCGGCCGCCGTTGGCGGTGAGCG and its reverse complement to obtain pIRESneoAdaptorMT.

EMSA³. p53 and its homologues were generated by *in vitro* transcription and translation using T7 RNA polymerase and rabbit reticulocyte lysate (Promega). These preparations (2.5 μl) were incubated with 1 μl of sheared salmon sperm DNA (0.1 μg/μl), 1 μl of poly(deoxyadenylate-deoxythymidylic acid; 1 μg/μl), and 7 μl of EMSA buffer [25 mM Tris-Cl (pH 7.5), 130 mM NaCl, 3 mM KCl, 5% bovine serum albumine, 12% glycerol, and 1 mM DTT] for 10 min at 23°C. Subsequently, 1 ng of the radiolabeled DNA probe was added. The probes were generated by annealing the indicated oligonucleotides to each other and performing a fill-in reaction using *exo*⁻ Klenow enzyme (MBI Fermentas) and α-³²P-dCTP. The incubation was then continued for 1 h at 23°C. The reaction mixes were separated at 4°C on a native 5% polyacrylamide gel with 0.5× Tris-borate EDTA (10× Tris-borate EDTA: 890 mM Tris base, 890 mM boric acid, and 20 mM EDTA) as running buffer, followed by autoradiography using a Bioimager (Fuji). The oligonucleotides

Received 11/18/02; accepted 6/2/03.

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¹ This work was supported by the German Research Foundation, the Deutsche Krebs-hilfe/Dr. Mildred Scheel Stiftung, and the P. E. Kempkes Foundation. A. C. received the fellowship BD 21601/99 from FCT, Portugal, and P. K. was supported by the German National Merit Foundation during this work.

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³ The abbreviations used are: EMSA, electrophoretic mobility shift analysis; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorting; MOI, multiplicity of infection.

had the following sequences: p21 forward: GATCGCGGCCGGAACATGTCCCAACA; p21 reverse: GGGCAACATGTTGGGACATGTTTCGCGGC-CGC; p21 mutant forward: GATCGCGGCCGCGAAAAATTTCCCAAAA; and p21 mutant reverse: GGGCAAAAATTTGGGAAAATTTTCGCGGCCGC.

Colony Forming Assays. Cells were transfected with plasmids to express bicistronic mRNAs encoding the adaptor and a neomycin resistance. The cells were subjected to selection by adding G418 in cell culture flasks. After 3 weeks, the emerging colonies were fixed and stained with crystal violet. The flasks were directly scanned to obtain digital images. These were used to calculate the area of the flask that was covered with cells. This number was set 100% for the mutant adaptor, and the values obtained with the nonmutant adaptor were normalized accordingly.

Recombinant Adenoviruses. The shuttle plasmid pAdtrackCMVAdaptor was constructed by cloning the insert of pcDNA3adaptor into pAdtrackCMV (8) with *Bam*HI and *Xba*I. The p53-coding region was excised from the plasmids pRcCMVp53 and pRcCMVp53R273H (9) with *Xba*I and *Hind*III and ligated into pAdtrackCMV after digestion with the same enzymes. Recombinant adenovirus was generated in all cases by homologous recombination to the plasmid pAdEasy1 (8) in *Escherichia coli* and transfection of HEK293 cells. Viruses expressing solely the GFP or β -galactosidase were generated in parallel. Viruses were amplified, and titers were determined as described previously (10–13).

Immunoblots. Proteins were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose, followed by incubation with antibodies in PBS containing 5% milk powder and Tween 20 (0.05%) and chemiluminescent detection (Pierce) of peroxidase-coupled secondary antibody against mouse IgG (Jackson). Antibody Ab-1 to p21/cip1/waf1 was from Calbiochem. The monoclonal antibody 2A10 against mdm2 was a generous gift of Arnold J. Levine.

FACS Analysis. After transduction and incubation, 2×10^5 cells were harvested by combining the detached cells in the supernatant with the adherent cells from the same well that had been removed by trypsinization. The cells were pelleted and resuspended in 0.5 ml of PBS. They were fixed by slowly adding 70% (v/v) ethanol to a final volume of 2 ml, followed by overnight incubation at 4°C. The cells were pelleted again and resuspended in 100 μ l of PBS containing RNase I (1 mg/ml). After overnight incubation at 4°C, the cells were stained by adding 15 μ l of propidium iodide (1 mg/ml) and 400 μ l of PBS. The intensity of staining was determined for at least 25,000 cells in each assay, using a FACSCalibur Flow Cytometry System (Becton Dickinson).

Results and Discussion

To create an adaptor molecule that simultaneously binds p53-responsive promoter DNA and mutant p53, portions of p53 and its homologue p73TA β were fused to each other. To achieve this, the coding region for the p53 oligomerization domain (residues 313–364 of human p53) was cloned upstream of the coding sequence of the central and COOH-terminal regions of p73TA β (residues 30–499 of human p73TA β). The encoded fusion protein contains no endogenous transactivation domain; rather, it contains the domains of p73TA β that are required for DNA binding, and these domains are fused at the NH₂ terminus to the oligomerization domain of p53 (Fig. 1A). Accordingly, EMSAs showed that the adaptor formed a specific complex with a p53-responsive DNA element (Fig. 1B).

While interacting with p53-responsive promoter DNA, the p53/p73 chimera can be expected to bind mutant p53 molecules. We propose that four adaptor molecules form a tetramer that associates with one p53-responsive promoter. The model additionally suggests that each of these four adaptor molecules will associate with up to three mutant p53-molecules because the p53-derived oligomerization domains will form a tetramer as well. These considerations suggest that up to 12 mutant p53-molecules could be tethered to a p53-responsive promoter by the adaptor to activate transcription (Fig. 1C). To test the function of the p53/p73 chimera (referred to as the adaptor from here on), it was transiently expressed in the cell line H1299 that carries no endogenous p53. When the p53-mutant R273H was coexpressed, p53 activity was readily measurable by a p53-inducible luciferase reporter

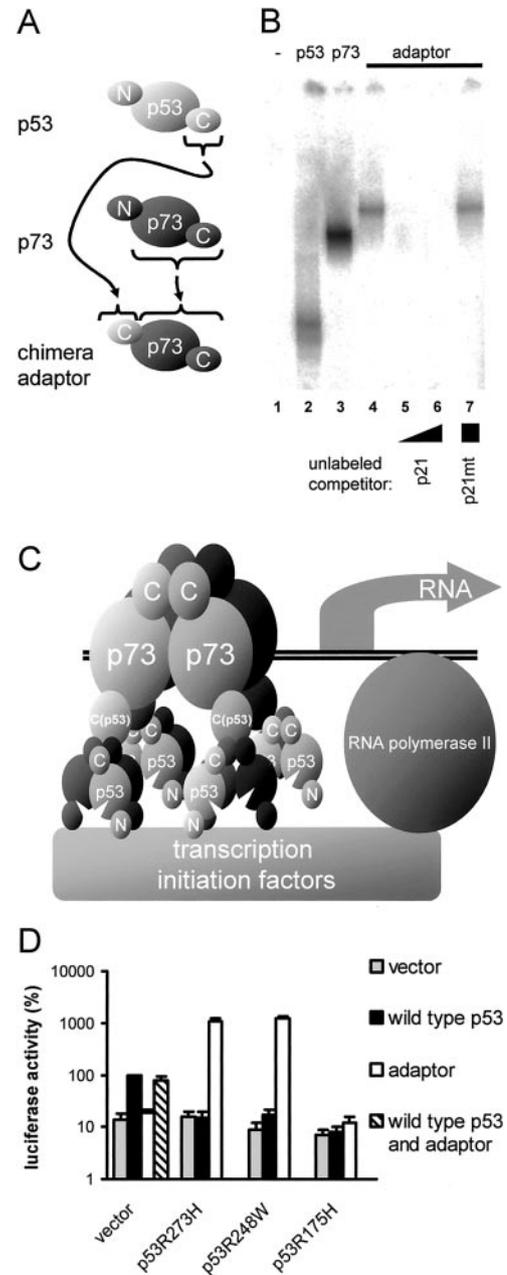


Fig. 1. A chimera of p53 and p73 that binds p53-responsive promoter DNA and cooperates with mutant p53 to activate transcription. **A**, schematic presentation of the adaptor. The COOH-terminal oligomerization domain of p53 was fused to the central portion of p73's NH₂ terminal transactivation domain. **B**, the adaptor specifically interacts with p53-responsive DNA. A radiolabeled portion of the p21 promoter was incubated with reticulocyte lysates programmed with an empty vector plasmid (1) or with expression plasmids for p53 (2), p73 (3), or the adaptor protein (4–7). In *Lanes* 5 and 6, the same DNA was added without radioactive label in 10-fold or 100-fold excess, respectively. In *Lane* 7, a mutant DNA that does not bind p53 was added at 100-fold excess. Note that the nonbound DNA was allowed to run out of the bottom of the gel, as to separate protein-DNA complexes of different sizes. **C**, a model of the adaptor function. The adaptor protein oligomerizes and binds DNA but does not contain a functional transactivation domain. Instead, it is expected to recruit mutant p53 to p53-responsive promoters, thereby tethering transcription initiation factors (such as TFIID and p300/CBP) and ultimately RNA polymerase II to the vicinity of these promoters. Note that the stoichiometry of adaptor molecules versus p53 mutants is not necessarily as depicted in the model, but in any case, the number of recruited p53 molecules is sufficient to allow transcriptional activation (see *D*). **D**, transcriptional activation by the adaptor and mutant p53. Expression plasmids for wild-type p53 (10 ng) or the adaptor protein (10 ng), or both (10 ng each) were transfected into H1299 cells along with expression vectors for the indicated p53 mutants (2.3 μ g each) and a p53-responsive luciferase reporter plasmid (100 ng; *mdm2*-promoter in pGL3, Promega). Twenty-four h later, the cells were harvested, and luciferase activity was determined. The results of at least three independent experiments are shown along with the SE. The value obtained with p53 alone was set to 100%, and the other values were normalized accordingly. Note that the results are shown on a logarithmic scale.

(Fig. 1D). On the other hand, the adaptor did not activate transcription on its own. Importantly, the adaptor did not increase the activity of wild-type p53 either, perhaps because wild-type p53, when not bound to DNA, assumes a conformation that compromises the function of the transactivation domain (14). In the presence of coexpressed mutant p53, the adaptor not only yielded high reporter activity, but the observed activity was also far stronger than with the combination of wild-type p53 and mutant p53. The reason is presumably that the DNA binding activity of the adaptor is not inhibited by the *trans*-dominant negative effect of mutant p53. Three different p53 mutants were tested in this system (Fig. 1D). The p53 mutants R273H and R248W [both class I-mutants (15)] could be activated by the adaptor, whereas the mutant R175H (class II) was not responsive to it. This is in agreement with previous reports that describe the association of mutant p53 with the Gal4 DNA binding domain: these chimeras also led to the activation of Gal4 reporter constructs when the p53 mutation was of class I but not class II (Refs. 14, 16 and our unpublished observations). To exclude the possibility that the p53 oligomerization moiety of the adaptor alone might be responsible for activation of mutant p53, a stop codon was inserted into the adaptor expression construct, resulting in expression of the p53 oligomerization domain without the p73 domains. When this construct was cotransfected with expression vectors for p53 mutants, this did not result in promoter activation (data not shown). Taken together, these results show that the adaptor protein can serve as an efficient and specific activator of class I p53 mutants. The relative frequencies of p53 mutations at residues 273, 248, and 175 are 11, 10, and 7%, respectively (15). Thus, the adaptor has the potential to reactivate the two most frequent p53 mutants, 273 and 248, which together are found in ~20% of all tumors with p53-mutations or in 10% of all human malignancies. Therefore, at least conceptually, a considerable number of tumors would be amenable to treatment with the adaptor protein. However, this would require precise diagnostics of the presence and kind of a p53 mutation, *e.g.*, through the use of DNA chips (17).

To test its function as a potential therapeutic, the adaptor was transiently expressed in tumor-derived cell lines that contain endogenous mutant p53. In these cell lines, the adaptor enhanced the expression of a p53-responsive reporter construct. In contrast, no promoter activation was observed in cells that contain no p53 or wild-type p53 (Fig. 2A).

Next, we tested the impact of the adaptor on the growth of tumor cells. Plasmids were engineered to express a bicistronic mRNA that encodes the adaptor protein and a mediator of neomycin resistance. As a control, a mutant adaptor was used that carries a mutation within the p53 oligomerization domain, thereby abolishing the formation of dimers or tetramers. C33A cells, a human cervical carcinoma-derived cell line that carries the p53-mutation R273C, were transfected with these plasmids, and colony formation was monitored under selection with G418 (Fig. 2B). The number of colonies was strongly suppressed when the adaptor protein was expressed, as compared with the inactive adaptor. When this assay was carried out using H1299 cells, derived from a human lung adenocarcinoma with no endogenous p53, no reduction in colony number was observed. Digital quantification of the area covered by colonies confirmed these results (Fig. 2C). Thus, the adaptor specifically suppresses the growth of cells that contain mutant p53.

To express the adaptor uniformly and efficiently, an adenovirus construct was made using a plasmid-based system [AdEasy (8)]. In parallel, adenovirus vectors were created to express wild-type p53, the p53 mutant R273H, and the GFP. H1299 cells were transduced with these vectors, and the expression of the p53-responsive genes *p21* and *mdm2* was assessed by immunoblot detection of their products. In the presence of coexpressed p53R273H, *p21* and *mdm2* levels were

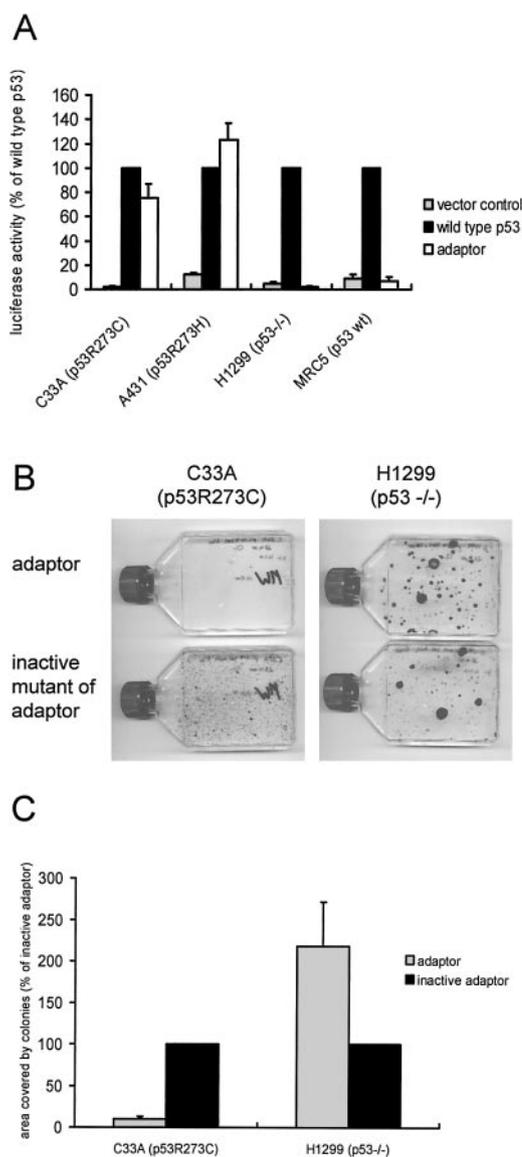


Fig. 2. Reactivation of endogenous mutant p53 and suppression of cell growth by the adaptor. **A**, activation of a p53-responsive promoter. The indicated cell lines were transfected with an empty vector plasmid or expression plasmids for p53 or the adaptor, 100 ng each, and a p53-responsive reporter plasmid (as in Fig. 1D, 100 ng). The total amount of transfected plasmid was brought to 2.4 μ g with pcDNA3 (Invitrogen). Luciferase activity was determined as in Fig. 1D. The value obtained with p53 was set to 100% for each cell line, and the other values were normalized accordingly. **B**, suppression of colony formation by the adaptor. C33A cells and H1299 cells were transfected with bicistronic expression plasmids for the adaptor or an inactive mutant that lacks a functional p53 oligomerization domain (see "Materials and Methods"). Stable transfectants were grown to colonies and stained with crystal violet 3 weeks after transfection. **C**, the stained flasks were transformed into digital images, followed by quantification of the area covered by colonies. The values obtained with the mutant adaptor were set to 100%, and the other values were normalized accordingly. The mean and SE of at least three independent experiments is shown in each case.

increased by the adaptor far more efficiently than by wild-type p53 (Fig. 3A). When the adaptor was introduced by adenovirus transduction into cell lines containing mutant p53, it increased the levels of *p21* and *mdm2* to an extent comparable with wild-type p53, but this was not observed in cells that contain endogenous wild-type p53 (Fig. 3B). On the contrary, in the latter cells, the adaptor moderately reduced the expression of *p21* (Fig. 3B, Lane 15), possibly through competition with endogenous wild-type p53 for promoter binding sites. Thus, when transduced by an adenovirus vector, the adaptor protein is capable of inducing p53-responsive genes specifically in tumor cells that contain mutant p53.

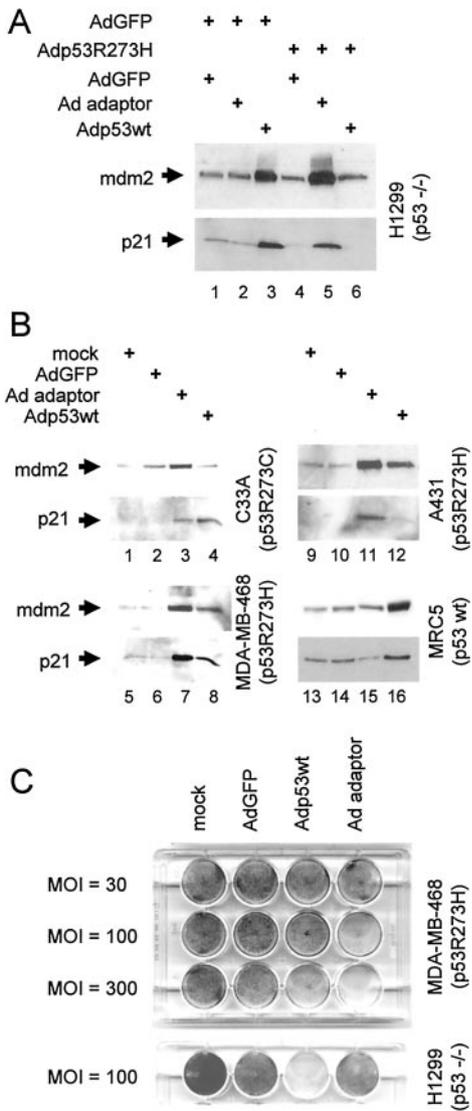


Fig. 3. Induction of p53-responsive genes and detachment in tumor cells with mutant p53 by expression of the adaptor through an adenovirus vector. **A**, induction of p53-responsive genes by expression of the adaptor and mutant p53 in combination. H1299 cells were transduced with recombinant adenoviruses that express the GFP or the p53 mutant R273H at a MOI = 100 and simultaneously with adenoviruses that express GFP or the adaptor protein or wild-type p53 (MOI = 10). Twenty-four h later, the cells were harvested, and mdm2 and p21 were detected by Western blot analysis. **B**, induction of p53-responsive genes by the adaptor in the presence of endogenous mutant p53. The indicated cell lines were mock infected or transduced with recombinant adenoviruses to express GFP or the adaptor protein or wild-type p53 (MOI = 100), followed by Western blot detection of mdm2 and p21. **C**, induction of cell detachment by the adaptor in the presence of endogenous mutant p53. MDA-MB-468 cells, containing mutant p53, and H1299 cells, lacking p53, were transduced in 12-well dishes as indicated. Four days later, detached cells were washed off, and the remaining cells were stained with crystal violet.

Finally, the adaptor protein mediated the detachment of tumor cells containing mutant p53 when expressed by a recombinant adenovirus (Fig. 3C), and the removal of cells appeared even more efficient than by the expression of wild-type p53. In contrast, cells that lack p53 were removed by adenovirus-expressed wild-type p53 but not by the adaptor.

To evaluate the induction of apoptosis in a quantitative fashion, tumor cells were transduced with adenovirus vectors to express the adaptor protein or wild-type p53. At different time points, the cells were analyzed by FACS, and the percentage of cells with a sub-G₁ DNA content was assessed. This number was used to determine the extent of apoptosis. It was found that both p53 and the adaptor protein induced apoptosis in tumor cells, although the effect of the adaptor

was delayed and not as extensive when compared with wild-type p53. However, apoptosis induction by the adaptor was specific for C33A cells that contained mutant p53 in contrast to H1299 cells lacking p53, whereas wild-type p53 induced death in all of the cells under study (Fig. 4).

Our results demonstrate that, in principle, an adaptor protein can be used to efficiently reactivate mutant p53. This one-hybrid system might represent an attractive alternative to the reintroduction of wild-type p53. It avoids the *trans*-dominant negative effect of endogenous mutant p53 such as other p53 derivatives that have modified COOH-terminal domains (18), but on top of this, it is specific for tumor cells with mutant p53. This specificity of cell elimination may allow the application of higher doses, as compared with the previously described p53 vectors.

In a different approach to restore p53 activity in tumor cells, it has been attempted to reactivate the mutant p53 protein by small drug candidates (19, 20). These compounds were shown to shift the conformation of the p53 tetramer and sometimes partially re-enable mutant p53 to activate promoters. However, at least thus far, these

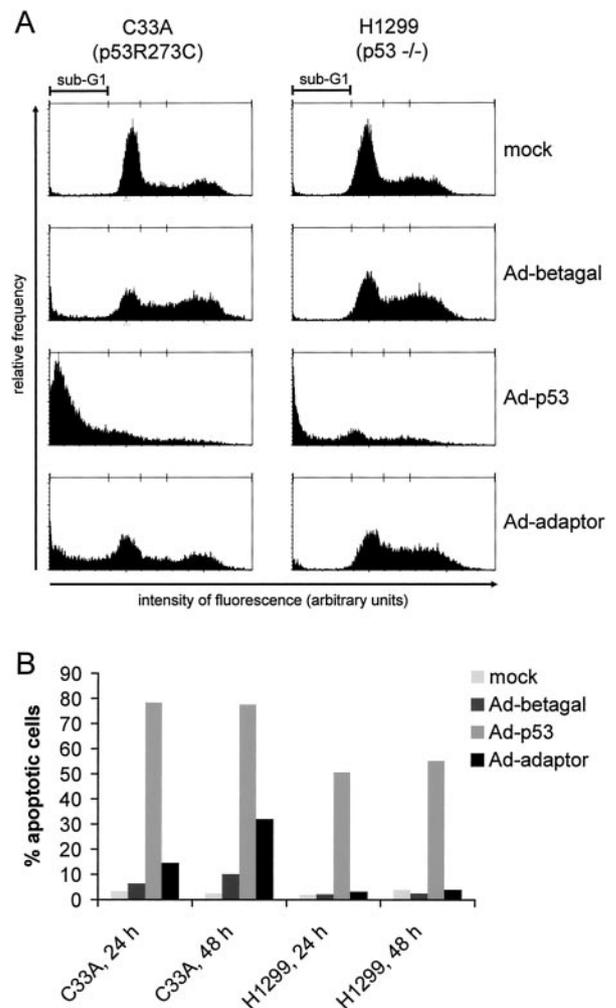


Fig. 4. Induction of cell death in tumor cells with mutant p53 by expression of the adaptor through an adenovirus vector. **A**, H1299 and C33A cells were transduced with adenovirus vectors to express β -galactosidase (control), p53, or the adaptor (MOI = 100), or mock infected. 48 h later, the cells were fixed, stained with propidium iodide, and analyzed by FACS. The distribution of frequencies of the fluorescence signals (corresponding to DNA content) is shown in each case. Note that adenovirus vectors generally appear to increase the frequency of cells with a DNA content greater than G₁. We propose that this might be attributable to the fluorescence derived from virus genomes. **B**, the percentage of cells with a DNA content corresponding to less than G₁ cells (sub-G₁ shoulder) was determined at 24 and 48 h after transduction.

classes of compounds were not efficient enough to be taken to the clinics. Therefore, the use of gene therapy to reactivate mutant p53 may deserve additional evaluation.

Acknowledgments

We thank Hans-Dieter Klenk and Rudolf Arnold for continuous support. We thank Arnold J. Levine and Bert Vogelstein for the generous gift of plasmids, antibodies, and cells. We also thank Jane Flint for antibodies and Daniel Caput for plasmids; Wolfgang Deppert and Silke Dehde for early passage H1299 cells; and Tanja Strive and Klaus Radsak for MRC5 human fibroblasts. We also thank Martin Eilers for helpful discussion and for providing cytometry equipment.

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