Reactivation of Mutant p53 by a One-Hybrid Adaptor Protein

Judith Roth, Claudia Lenz-Bauer, Ana Contente, Kristina Löhrl, Philipp Koch, Sandra Bernard, and Matthias Dobbelstein

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, 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 transcriptional 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

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

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had the following sequences: p21 forward: GATCGCGCCGCGAAAATTCCTCCAAA; p21 reverse: GGCGCAAAATTTGAAAAATTTTCCGGCAGC; p21 mutant forward: GATCGCGCCGCGAAAATTCCTCCAAA; and p21 mutant reverse: GGCGCAAAATTTGAAAAATTTTCCGGCAGC.

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 colonies 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 BamHI and XhoI. The p53-coding region was excised from the plasmids pRcCMVp53 and pRcCMVp53R273H (9) with XhoI and HindIII 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 μl of PBS containing RNase I (1 mg/ml). After overnight incubation at 4°C, the cells were stained by adding 15 μl of propidium iodide (1 mg/ml) and 400 μl 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 p73TAβ 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 p73TAβ (residues 30–499 of human p73TAβ). The encoded fusion protein contains no endogenous transactivation domain; rather, it contains the domains of p73TAβ 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
DNA binding activity of the adaptor is not inhibited by the wild-type p53 and mutant p53. The reason is presumably that the observed activity was also far stronger than with the combination of p53, the adaptor not only yielded high reporter activity, but the transactivation domain (14). In the presence of coexpressed 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 p53 mutant 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, thereby abolishing the formation of dimer 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. 2A). The value obtained with p53 was set to 100% for each cell line, and the other values were normalized accordingly. The mean and SE of at least three independent experiments is shown in each case.

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 dimer 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 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.
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
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.

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