Cells with Defective p53-p21-pRb Pathway Are Susceptible to Apoptosis Induced by p84N5 via Caspase-6

Elizabeth Garner,1 Fabio Martinon,2 Jurg Tschopp,3 Peter Beard,4 and Kenneth Raj1

1Department of Virology, National Institute for Medical Research, London, United Kingdom; 2Department of Immunology and Infectious Disease, Harvard School of Public Health, Boston, Massachusetts; 3Institute of Biochemistry, University of Lausanne; and 4Swiss Institute for Experimental Cancer Research (ISREC), Epalinges, Switzerland

Abstract

Adeno-associated virus (AAV) infection triggers a DNA damage response in the cell. This response is not induced by viral proteins but by virtue of the structure of AAV ssDNA being recognized by the cell as damaged DNA. The consequence of this is the killing of cells lacking p53 activity. We have observed that cells that lack p21 or pRb activity are also sensitive to AAV-induced cell death. We report that cells respond to AAV infection by activating two DNA damage signaling cascades. The first activates the p84N5 protein, which in turn activates caspase-6, leading to cell death. The second cascade activates the p53-21-pRb pathway, which inhibits activation of the p84N5 protein and thus prevents cell death. The result of the antagonistic interaction between these two pathways is that cells that do not exhibit functional p53-p21-pRb signaling undergo apoptosis as a consequence of AAV infection. Cells with a functional p53-21-pRb pathway are refractory to AAV-induced cell death. These results show that p53, although a proapoptotic protein, together with pRb and p21 proteins, is a member of an antiapoptotic cellular mechanism. As such, these experiments reveal features that may be exploited to specifically kill cells that lack the p53-p21-pRb pathway, such as cancer cells. The use of AAV to exploit these subtle characteristics of intracellular signaling further highlights the advantages of using viruses as precision tools with which to address questions of cell biology. [Cancer Res 2007;67(16):7631–7]

Introduction

The ability of p53 to stimulate apoptosis and cell cycle arrest in the event of DNA damage is well documented. The p53 protein is defective in approximately half of all cancers as is the downstream recipient of p53 signaling, pRb. Together, the p53-p21-pRb signaling pathway is defective in the majority of cancers. The absence of this pathway confers growth advantage because it allows cells to avoid signals that might otherwise result in cell cycle arrest or apoptosis. Although the absence of the p53-p21-Rb pathway is undoubtedly advantageous to the cell in terms of proliferation, our recent observation suggests that, in some instances, it may also be detrimental. Our investigations have centered on examining the effects that adeno-associated virus (AAV) (1) has on the cells that it infects. We have reported that cells lacking p53 are susceptible to AAV-induced apoptosis whereas normal and primary cells are not (2). The mechanism for this selectivity was not known, except for the fact that neither newly synthesized viral proteins nor proteins that are part of the in-coming viral particle are involved. Instead, it is the structure of the viral DNA itself that causes G2 cell cycle arrest or cell death in the absence of p53 protein (2). Because selective elimination of cells lacking p53 activity is important with regard to cancer therapy, we have focused on understanding how AAV affects cells in this way. We observed that, on infection, the viral DNA is rapidly sequestered into large foci within the nucleus of the cell. Within these foci are many cellular proteins including ATR, Brca1, RPA, TopBP1, Rad17, and Rad51 (3). These proteins signal the presence of damaged cellular DNA and are mobilized into distinct foci when damaged DNA is sensed. The nature of these foci is not entirely clear, but they are considered to be sites of damaged DNA or regions where DNA repair occurs, or both. Hence, the accumulation of these proteins into foci on AAV infection and the presence of AAV DNA within them are consistent with AAV DNA being recognized by the cell as damaged DNA (2, 3). This is a consequence of the fact that the AAV genome is a piece of ssDNA with both ends folded back to resemble hairpins (1, 4). As a result of being recognized by cellular DNA damage response proteins, a series of signaling cascades are activated in response to AAV DNA that cause the activation or inactivation of various cellular regulatory proteins. The outcome of such signaling is greatly influenced by the presence or absence of p53 activity. The infection of a cell with functional p53 protein results in p53 activation, followed by an increase in the level of the p21 protein and the rapid degradation of the Cdc25C phosphatase. A consequence of this is the inability to activate cyclin B-Cdc2, resulting in the arrest of cells in the G2 phase of the cell cycle. Contrary to expectation, cells that lack p53 activity are killed on AAV infection (2). This outcome is counterintuitive because the p53 protein has extensively been studied and shown to be predominantly proapoptotic (5–7). Here, we address the question of why cells that do not express functional p53 are susceptible to AAV-induced cell death and how the activity of p53 may prevent such apoptosis. The data presented here show that it is not only the absence of p53 that predisposes AAV-infected cells to cell death but also the absence of p21 and pRb proteins.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancercres.aacrjournals.org/).

Requests for reprints: Kenneth Raj, Department of Virology, National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA United Kingdom. Phone: 44-20-8816-2191; Fax: 44-20-8906-4477; E-mail: kra@nimr.mrc.ac.uk.

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the event of DNA damage signaling. This feature may be exploited for cancer therapy because this pathway is frequently defective or compromised in cancer cells.

Materials and Methods

Cell lines, transfection, infection, and plasmids. Saos-2 cells, U2OS cells, and HCT116 cell line (a kind gift from B. Vogelstein, The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Selection of transfected cells was done with 4.5 µg/mL puromycin, 5.0 µg/mL blasticidin, or 500 µg/mL neomycin. Transfections were done using Effectene (Qiagen) according to the manufacturer’s instructions. Plasmids expressing dominant negative caspase-6 and dominant negative caspase-3 were kindly provided by D.E. Bredesen (Buck Institute, Novato CA). For infection of U2OS and Saos-2 cells, a multiplicity of infection of between 2,000 and 5,000 was used. Cells in 10-cm dishes were infected with AAV in a volume of 2 mL of DMEM with 10% FBS for 3 h before addition of fresh medium up to 10 mL.

Fluorescence-activated cell sorting analysis of DNA content. Cells were harvested by trypsinization and fixed in 70% ethanol. Cells were pelleted and resuspended in RNase A (0.1 mg/mL in PBS) and incubated for 10 min at 37°C. One volume of 40 µg/mL propidium iodide solution in PBS was then added. DNA content was analyzed using FACS and CellQuest software (Becton Dickinson). A total of 10,000 cells were counted for each analysis.

Methylene blue staining of tissue culture cell monolayers. Cell monolayers were washed twice in PBS, following which a solution of 1% (w/v) methylene blue in 50% methanol/PBS was overlaid onto cells. Dishes were incubated at room temperature for 30 min. Cells were then washed thoroughly with PBS and air-dried in the dark.

Vectors and retrovirus production. For siRNA expression silencing of pRb, the sequences 5′-GATCCCCGCGCATTGGAATGTTAGCTTCAAGAGAGAC TAAACATTTCAAGTGCTTTGGAAA-3′ and 5′-AGCTTTTC-CAAAAGCCACTTGAAATGTTAGTCTCTTGAAGACTAACATTT-¶/C2/¶-GATCCCCGCCACTTGAAATGTTAGTCTTCAAGAGA-CAGTAAACATTTCAAGTGCTTTGGAAA-3′ and 5′-AGCTTTTC-CAAAAGCCACTTGAAATGTTAGTCTCTTGAAGACTAACATTT-¶/C0/¶-GATCCCCGCCACTTGAAATGTTAGTCTTCAAGAGAGAC TAAACATTTCAAGTGCTTTGGAAA-3′ were annealed and cloned into the EcoRI site of the pSuper expression vector (kind gift from Thijs Brummelkamp, Whitehead Institute, Cambridge, MA). The siRNA insert and promoters were excised from pSuper using the Xho1 and EcoRI restriction sites and ligated into the pRetroSuper retroviral expression vector to yield the pRshshRb construct.

NXA cells (a kind gift from G. Nolan, Department of Microbiology and Immunology, School of Medicine, Stanford University, Stanford, CA) were transfected with the pRshshRb vector or an irrelevant (scrambled) sequence. Virus particles harvested 48 h later were supplemented with 10 µg/mL polybrene and used to infect a 10-cm tissue culture dish of cells. Twenty-four hours later, cells were selected with 1.5 µg/mL puromycin. To reconstitute p21 or pRb protein in Saos-2 cells, the pBabe Puro vector harboring these genes was used to generate retroviruses as above and used to transduce Saos-2 cells, which were then selected by puromycin selection.

The pB4ADD sequence was excised from the PCRI3 and inserted via EcoRI restriction sites into the pBabeBlast retroviral vector. pNXA cells were transfected with this construct as delineated above to generate retroviruses. Cells expressing the dominant-negative construct or the empty vector control were selected using blastidin (7.5 µg/mL).

Western blotting and protein analysis. Cells were washed in PBS, harvested by scraping, pelleted, and then resuspended in Reporter lysis buffer (Promega) with a cocktail of protease inhibitors (Calbiochem) and sonicated on ice. Lysates were centrifuged at 16,000 × g for 5 min and the supernatant collected. Equal amounts of protein were loaded onto SDS-polyacrylamide gels and transferred onto nitrocellulose membranes and analyzed with antibodies to p53 (Novocastra, NCL-p53-D01), phospho-Ser15 p53 (Cell Signaling), p21 (Santa Cruz Biotechnology), pRb (BD PharMingen), actin (Santa Cruz Biotechnology), caspase-3 and caspase-6 (Cell Signaling), or p4N5 (BD Transduction).

Results

Virus inactivation and cell infection. AAV, prepared as described (29), was plated with PBS to a total volume of 10 µL in a four-well dish and exposed to 3,000 J/m2 of UV irradiation. Viruses were then resuspended in 3-mL DMEM and overlaid onto cells. Five hours postinfection, medium was added to a total volume of 10 mL.

AACR Research.

AAV arrests cells in S2 phase and induces death of cells that lack p53 or p21 proteins. We have previously shown that the outcome of AAV infection is dependent on the presence of functional p53 protein. The presence of wild-type p53 results in the infected cells arresting in the G2 phase of the cell cycle followed by cell cycle re-entry ~3 days postinfection. Conversely, the absence of p53 activity results in cell death (2, 3). Figure 1A shows representative fluorescence-activated cell sorting (FACS) analyses of AAV-infected cells to show the premise on which the subsequent experiments reported here are based. U2OS (p53+/−) and Saos-2 (p53−/) are osteosarcoma cells, whereas HCT116 p53+/+, p53−/−, or p21−/− are colon carcinoma cells (14). FACS analyses of additional cell types with reduced or absent expression of different genes, together with the deduced pathways of DNA damage signaling and cell cycle arrest, have previously been described (2, 3). The representative data in Fig. 1A serve to emphasize that the observed cellular responses to AAV are not cell type specific and occur in multiple cell types. Figure 1A also shows that although p53 is important in determining the outcome of AAV infection, the downstream transcriptional target of p53, p21, is similarly important. This is highlighted by the observation that HCT116 p21−/− cells, despite expressing functional p53 protein, also undergo apoptosis following AAV infection.

Increased p21 protein levels alone cannot protect cells from AAV DNA−induced apoptosis. The activation of p53 on AAV infection can be assayed by examining levels of p53 Ser15 phosphorylation. As indicated in Fig. 1B, the level of Ser15 phosphorylation increases on AAV infection, thus confirming activation of p53. This activation is accompanied by an increase in the level of the p21 protein, whose synthesis is profoundly stimulated by p53 (2). We have previously observed that although other p53 targets (such as 14-3-3ζ; ref. 15) also increase, it is the increase of p21 protein that is crucial in preventing cell death on AAV infection. To ascertain whether elevated expression of p21 would rescue Saos-2 cells from AAV-induced apoptosis, Saos-2 cells were transduced with recombinant retroviruses carrying the p21 gene. The amount of p21 protein in transduced cells was greatly augmented when compared with control cells that were transduced with the empty retrovirus vector (Fig. 1C). In spite of this, cells expressing elevated levels of p21 were not protected from death when infected with AAV (Fig. 1D). FACS data to show cell death is accompanied by data from long-term survival assays. Long-term survival assay data are shown because it has been highlighted that short-term assays for cell death can be misleading as they only measure the rate of apoptosis as opposed to true resistance. As such, long-term survival assays are considered the most reliable test of true resistance to cell death (16). Initially, the data shown in Fig. 1 seem to disprove the notion that p21 protects cells from cell death when infected with AAV. However, it is important to consider that one of the major downstream targets of p21 (via cyclin/cyclin-dependent kinases) is also absent in Saos-2 cells, the tumor suppressor protein pRb. We hypothesized that protection from AAV-induced cell death may require signaling to
be conveyed via p53, p21, and ultimately to pRb (by the inhibition of cyclin/cyclin-dependent kinase; ref. 17).

**AAV infection activates pRb protein.** If the above hypothesis is correct, then cells resistant to AAV-induced cell death should exhibit activation of pRb following AAV infection. To examine this, we used immunoblotting to analyze the pRb protein in lysates of U2OS cells that were infected with AAV. It was evident from its accelerated migration through the gel that pRb was predominantly of the hypophosphorylated form (Fig. 2A), indicating that pRb was activated on AAV infection. Clearer evidence of pRb dephosphorylation is illustrated in the independent experiment shown in Fig. 2B. The activation of pRb in U2OS cells on AAV infection reinforces the possibility that pRb may be important in terms of protection from AAV-mediated death in these cells.

**Increased pRb protein levels alone cannot protect cells from AAV DNA–induced apoptosis.** The observation that pRb may protect cells from AAV-mediated cell death suggests that the reconstitution of pRb in Saos-2 cells may confer protection. To test this hypothesis, recombinant retroviruses carrying the pRb gene were introduced into Saos-2 cells, which do not express wild-type pRb protein. Although the pRb protein was produced in readily detectable amounts in these engineered Saos-2 cells (Fig. 2C), this failed to prevent cell death following AAV infection (Fig. 2D). We then attempted to test the possibility that a combination of these proteins (p53, p21, and pRb) would be required for protection. However, it was not experimentally viable to generate Saos-2 cells that coexpress both p21 and pRb because the cells that survive following antibiotic selection no longer express these proteins (Supplementary Fig. S1A). Likewise, whereas it is possible to coexpress p53, p21, and pRb proteins transiently in Saos-2 cells, antibiotic selection results in no surviving cells (Supplementary Fig. S2). It is clear that the reintroduction of these proteins caused either cell death or cell cycle arrest of Saos-2 cells, thus excluding their use in subsequent experiments. Therefore, it was necessary to conduct alternative experiments to test the hypothesis that a combination of p53, p21, and pRb is necessary to protect cells from AAV-induced cell death.

**Removal of pRb abrogates resistance of cells against AAV.** To examine this hypothesis, we decided to disrupt the p53-p21-pRb pathway in cells that are resistant to cell death on AAV infection. Previously, we observed that when the p53 in U2OS cells was inactivated by dominant-negative p53 protein, cells underwent apoptosis when infected with AAV. Now we considered what would be the outcome if pRb protein levels were compromised in these cells. Expression of short hairpin RNA (shRNA) against pRb significantly reduced the level of pRb protein with no alteration in p53 protein level (Fig. 3A). Infection of these cells with AAV resulted in their death, as opposed to the resistance exhibited by control cells expressing scrambled shRNA in the survival assay (Fig. 3B). Together, these results suggest that all components of the p53-p21-pRb pathway are required to confer cellular resistance to AAV-induced cell death. On this notion, we proceeded to inquire how this pathway is linked to cell death.

**Caspases activated by AAV infection.** It is not immediately clear how p53 signaling that leads to the activation of pRb can prevent cell death induced by AAV DNA. To gain further insight into how this might occur, we decided to characterize the cell
was also activated (Fig. 4B). However, of particular interest was the observation that caspase-6 activation in AAV-infected Saos-2 cells, it is not in AAV-infected U2OS cells. Within the small panel of proteins known to activate caspase-6, there was the observation that caspase-6 was also activated (Fig. 4B). We analyzed the activation of caspase-6 in Saos-2 cells, U2OS cells, and U2OS cells expressing shRNA against pRb. As shown in Fig. 4B, whereas caspase-6 is clearly activated in AAV-infected Saos-2 cells, it is not in AAV-infected wild-type U2OS cells. However, when U2OS cells expressing shRNA against pRb were infected with AAV, caspase-6 was activated. These observations are consistent with the fact that U2OS cells lacking pRb die following AAV infection (Fig. 3). From the literature, it seems that caspase-6 is activated less frequently than caspase-3, caspase-8, or caspase-9, which suggests that caspase-6 is activated by fewer effectors of apoptosis. Although this observation is suggestive, it remained possible that caspase-6 activation may only be a consequence of cell death as opposed to being the mediator of cell death.

Caspase-6 is the major caspase responsible for the death of cells infected with AAV. To determine whether caspase-6 is the primary mediator of cell death, we examined the susceptibility of Saos-2 cells to AAV-induced cell death following inhibition of caspase-6. To address this, we first expressed a flag-tagged dominant-negative caspase-6 point mutant in Saos-2 cells (18). As such, this protein appears as a slower-migrating protein band on the Western blot (Fig. 5A). In parallel, we also generated cells that expressed a point mutant of caspase-3, which has been shown to exhibit a dominant-negative effect on the wild-type caspase-3 protein. This dominant-negative protein has the same molecular weight as the wild-type, and hence the Western blot analysis with caspase-3 antibody reveals a significantly greater quantity of caspase-3 protein (Fig. 5A). Following infection with AAV, it is clear that inactivation of caspase-6 conferred considerable protection to the cells against AAV infection (Fig. 5C). Importantly, the degree of protection afforded by dominant-negative caspase-6 was even greater than that afforded by dominant-negative caspase-3 (Fig. 5B). In an independent experiment in which dominant-negative caspase-6 or dominant-negative caspase-3 was expressed in Saos-2 cells, the relative amounts of cell death following AAV infection were quantified following FACS analysis (Supplementary Fig. S3C). Approximately 90% of Saos-2 cells were observed to undergo apoptosis following AAV infection as shown by the percentage of cells with sub-G1 DNA content. However, only 20% of dominant-negative caspase-6 cells underwent cell death following AAV infection as opposed to 30% of dominant-negative caspase-3-expressing cells. This further reinforces the observation that caspase-6 is the major caspase involved in killing AAV-infected cells. Within the small panel of proteins known to activate caspase-6 is a protein known as p84N5 (10). The literature associated with p84N5 made this protein an attractive candidate mediator of AAV-induced apoptosis because p84N5 has been shown to be functionally associated with the activation of caspase-6 and, furthermore, p84N5 activation is inhibited by active pRb (9, 12, 13). The combination of literature reported activities of p84N5, and our observations thus far led us to hypothesize that p84N5 protein may provide the link between p53-p21-pRb signaling and apoptosis.

AAV activates p84N5. To ascertain whether p84N5 protein was affected in cells following AAV infection, lysates from AAV-infected Saos-2 and U2OS cells were immunoblotted with p84N5 antibodies. The p84N5 protein was visibly cleaved in Saos-2 lysates but not in U2OS lysates (Fig. 6A). The gradual increase of p84N5 cleavage is also observed in an independent experiment showing
additional time points post infection (Fig. 6B). This cleavage is notable because it signifies the activation of the p84N5 protein, which was first described by Doostzadeh-Cizeron et al. (9). Because p84N5 was activated in Saos-2 cells, which lack p53 and pRb, it is clear that the p84N5 activation pathway is separate from and does not require the p53-p21-pRb pathway. Instead, pRb has been reported to prevent p84N5 activation (12). We observe that AAV infection activates p84N5 and this, in turn, may stimulate p84N5-mediated apoptosis. We also observe that cells with a functional p53-p21-pRb pathway do not show p84N5 activation upon AAV infection. Ultimately, these observations culminate in the hypothesis that a functionally complete p53-p21-pRb pathway do not show p84N5 activation.

Inactivation of p84N5 rescues cells from AAV-induced apoptosis. To ascertain whether p84N5 mediates cell death in response to AAV infection, we considered whether inactivation of p84N5 would rescue Saos-2 cells, which lack the p53-p21-pRb pathway, from AAV-induced apoptosis. Saos-2 cells were generated expressing a dominant-negative p84N5 protein from a recombinant retrovirus construct (Fig. 6C). The dominant negative protein, which lacks the five death domains at the COOH terminus, was previously characterized and shown to inactivate the endogenous wild-type p84N5 in a dominant negative way (9). Following antibiotic selection of the transduced Saos-2 cells, the cells were infected with AAV. Methylene blue staining of duplicate plates 7 days post AAV infection revealed that, whereas few control Saos-2 cells remained on the plates, Saos-2 cells expressing the dominant negative p84N5 fully populated the plates (Fig. 6D). This result shows that inactivation of p84N5 rescues Saos-2 cells from death upon AAV infection. In summary, when p84N5 activity is abrogated, cells that lack the p53-p21-pRb pathway, which would otherwise be susceptible to AAV-induced cell death, become resistant. This observation shows that p84N5 is indeed the apoptosis-triggering factor that is activated by AAV infection. Together, these results and those that we have previously published support the following model. AAV DNA, owing to its complex single-stranded structure, activates a DNA damage response in the cell. Among the numerous biochemical cascades that are triggered, one activates pRb and another activates p84N5. These two cascades dictate the fate of the cell. If the first cascade successfully activates pRb, it would prevent the activation of p84N5 and, as a consequence, prevent initiation of apoptosis. In the absence of pRb activation, p84N5 would be cleaved and activated, resulting in apoptosis via caspase-6 activity.

Discussion

When AAV infects a cell, it introduces its ssDNA into the nucleus. AAV alone does not replicate in cells (1) but the ends of the AAV genome, which resemble hairpins, and secondary structures that form from the ssDNA cause the cell to initiate a DNA damage response (3). This response results in G2 phase arrest, following which cells re-enter the cell cycle ~ 3 days postinfection (2). Cell cycle re-entry coincides with the degradation of the AAV DNA that was responsible for triggering the DNA damage response. Previously, we observed that cells that lack p53 activity or p21 protein do not emerge from G2 arrest to go back into cycle. Instead, following G2 arrest, cells undergo the process of apoptosis. We have previously elucidated the pathway that leads to the observed G2 arrest (2), and here we present data addressing how apoptosis occurs upon AAV infection of these cells.

It was evident from our previous work that although p53 activity protected cells from cell death following AAV infection, the presence of p21 protein is also necessary to prevent AAV-induced cell death (2). The observation that p21 may confer protection from apoptosis has been explored by several laboratories (19–23). In the work described above, we set out to identify the events downstream of p21 that are subsequently activated by this line of signaling. Our results show that pRb is the eventual recipient of the AAV-induced DNA damage signaling via p53 and p21. As a consequence, pRb becomes activated. The link between AAV-induced activation of pRb and protection from cell death was uncovered by our experiments, which showed that...
Another signaling cascade activates p84N5. In the absence of active p84N5, p84N5 is not functional due to inhibition by active pRb. In the absence of active pRb, this period for repair cannot thus facilitating cell cycle re-entry. In the absence of p53 signaling and p84N5 activation may be a mechanism that has evolved to inhibit apoptosis in the event of less severe DNA damage to allow time for cells to activate repair mechanisms, particularly interesting that p84N5 is expressed in high amounts in breast carcinoma cells (25). This may indicate that these cells are primed for apoptosis, but it might also mean that this protein is mutated in these cancers and its accumulation poses no threat to the cells, akin to mutated p53 protein. An interesting possibility is that a DNA damage response might be switched on in these cells. This interpretation stems from our observation that p84N5 protein levels increase upon the DNA damage response induced by AAV infection (Fig. 6B). Why cancer cells would trigger a DNA damage response is not clear, but recent reports show that this does occur (26–28). It may be due to abnormal DNA replication or reduced ability of cancer cells to repair DNA lesions that accrue during DNA replication.

The proapoptotic activities of the p84N5 protein have not been extensively investigated, but rather the involvement of p84N5 in aspects of RNA processing has been more widely detailed. It is extensively investigated, but rather the involvement of p84N5 in aspects of RNA processing has been more broadly detailed. It is particularly interesting that p84N5 is expressed in high amounts in breast carcinoma cells (25). This may indicate that these cells are primed for apoptosis, but it might also mean that this protein is mutated in these cancers and its accumulation poses no threat to the cells, akin to mutated p53 protein. An interesting possibility is that a DNA damage response might be switched on in these cells. This interpretation stems from our observation that p84N5 protein levels increase upon the DNA damage response induced by AAV infection (Fig. 6B). Why cancer cells would trigger a DNA damage response is not clear, but recent reports show that this does occur (26–28). It may be due to abnormal DNA replication or reduced ability of cancer cells to repair DNA lesions that accrue during DNA replication.

Our described check and balance interaction between p53 signaling and p84N5 activation may be a mechanism that has evolved to inhibit apoptosis in the event of less severe DNA damage to allow time for cells to activate repair mechanisms, following which the DNA damage signal may be switched off, thus facilitating cell cycle re-entry. In the absence of p53 signaling and thus activated pRb, this period for repair cannot

the characteristics of AAV-induced cell death are similar to those of apoptosis induced by the proapoptotic protein p84N5. Both AAV and p84N5 induce cells to arrest at the G2 phase of the cell cycle before apoptosis. Both induce cell death via caspase-6. Whereas p84N5 is activated by DNA damage response, AAV induces a DNA damage response. Our subsequent experiments described above revealed that AAV actually induces cell death by activating the p84N5 protein. As such, we propose the following model. Upon AAV infection, the cell initiates a DNA damage response that triggers several signaling cascades, one of which leads to the activation of the p53 protein, followed by an increase in the level of the p21 protein and the eventual activation of pRb. Another signaling cascade activates p84N5. In the presence of the former signaling cascade, p84N5 is not functional due to inhibition by active pRb. In the absence of active pRb, p84N5 is activated, leading in turn to the activation of caspase-6 and apoptosis. Hence, cells that lack any component of the p53-p21-pRb pathway are susceptible to being killed by AAV, whereas cells in which this pathway remains intact are not.

The notion that p53 is involved in preventing apoptosis will understandably seem to be contrary to the well-documented proapoptotic activity of this protein. Although it is beyond doubt that p53 is a potent inducer of apoptosis, and the mechanism of this induction has been well delineated, this does not exclude p53 from participating in the mechanism of check and balance described above. It is important to note that p53 has indeed been shown to show cell cycle arrest properties (via p21 induction) that are not necessary for its apoptosis-inducing function. In fact, our observations suggest the p21-inducing activity of p53 to be the very feature that participates in the prevention of apoptosis. Evidently, the proapoptotic propensity of p53 is more evident in standard experimental conditions than its antiapoptotic nature. When cells are exposed to the DNA-damaging treatments commonly used in the laboratory, many different types of DNA lesion are generated. It is possible that the quantity and types of lesion affect the outcome of the DNA damage response. It is conceivable that the p53 proapoptotic activity prevails when the levels of DNA damage are high or when the cell recognizes multiple types of DNA lesion. Conversely, if the lesion types are few, p53 may exhibit a more protective activity. If so, one would expect, as is the case, that the proapoptotic activity of p53 would be more frequently observed in laboratory or clinical settings in which the DNA-damaging treatments used generate many lesions of multiple types, even to the extent that cells die not only from a DNA damage signaling response but also from physical causes such as mitotic catastrophe (24). In contrast, DNA damage signaling induced by AAV does not involve damaging cellular DNA because it is the structure of the viral DNA alone that induces the damage response. As such, AAV is a more refined agent that can induce DNA damage signaling in cells and, as is shown here, is able to uncover subtleties in the DNA damage response that are obscured by alternate mediators of DNA damage that are less discrete in the types and quantity of DNA lesions that they introduce.

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Figure 6. A, Western blotting of lysates of Saos-2 or U2OS uninfected cells (c) and AAV-infected cells 2 d post infection with antibodies against p84N5 protein. B, p84N5 Western blot analysis of lysates from Saos-2 cells that were harvested at various days post AAV infection. C, Western blot analysis with antibodies against p84N5 of untransduced cells (c), cells transduced with empty vector (v), or cells transduced with vector expressing dominant-negative p84N5 protein (DNp84), which is a shortened form (lacking the death domains) of the wild-type protein. D, triplicate methylene blue staining of uninfected Saos-2 cells, Saos-2 cells transduced with empty vector, or Saos-2 cells expressing dominant-negative p84N5 protein, 10 d post AAV infection.
be established because p84N5 is not inhibited, the result of which is apoptosis. The experiments described here facilitate our understanding of how AAV can selectively mediate the death of cells lacking p53, p21, or pRb and show a role for p53 signaling in protecting cells from apoptosis. It is hoped that this newly described interaction may prove to be a useful addition to our knowledge as we continue to search for exploitable features of cancer cells.

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Acknowledgments

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