Induction and Bypass of p53 during Productive Infection by Polyomavirus

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Lytic infection by polyomavirus leads to elevated levels of p53 and induction of p53 target genes p21Cip1/WAF1 (p21) and BAX. This is seen both in polyomavirus-infected primary mouse cell cultures and in kidney tissue of infected mice. Stabilization of p53 and induction of a p53 response are accompanied by phosphorylation of p53 on serine 18, mimicking a DNA damage response. Stabilization of p53 does not depend on p19Arf interaction with mdm2. Cells infected by a mutant virus defective in binding pRb and in inducing G1-to-S progression show a greatly diminished p53 response. However, cells infected by wild-type virus and blocked from entering S phase by addition of mimosine still show a p53 response. These results suggest a role of E2F target genes in inducing a p53 response. Polyomavirus large T antigen coprecipitates with p53 phosphorylated on serine 18 and also with p21Cip1/WAF1. Implications of these and other findings on possible mechanisms of induction and override of p53 functions during productive infection by polyomavirus are discussed.

The tumor suppressor p53 plays a pivotal role in carcinogenesis and is the most frequently mutated gene in human cancers (31, 34). Most DNA tumor viruses have mechanisms to inactivate p53. The large T antigen (large T) of simian virus 40 (SV40) binds p53 and inactivates at least some of its functions (44). The E6 proteins of the highly oncogenic human papillomavirus type 16 (HPV-16) and HPV-18 promote the rapid degradation of p53 through a ubiquitin-dependent proteolytic pathway (47). The oncogenic human adenoviruses act to block p53 through dual functions of the E1B proteins acting directly on p53 and on its downstream targets (16).

Thus far, none of these mechanisms have been ascribed to the mouse polyomavirus. This is surprising in view of the efficiency and rapidity with which this virus induces tumors. p53 is not stably upregulated in polyomavirus tumors, and most tumor cell lines examined show a normal p53 response to DNA damage (18). Regulation of p53 occurs through a variety of mechanisms that may operate differently in different cell types. The possibility that polyomavirus may have some way of counteracting p53 in various target cells was tested by determining the effect of the absence of p53 on tumor induction by the virus. Tumors arose significantly more rapidly in p53−/− than in p53+/+ or p53+/− mice, supporting the generally held view that this virus has no effective way of blocking p53 functions during the course of tumor development (18). This result contrasts with those in the SV40 system, where the large T antigen clearly binds p53 and where the absence of p53 in the host can retard rather than accelerate tumor development (28).

A single cycle of polyomavirus growth in mouse cells requires roughly 48 h and is dependent on cell cycle progression from G0/G1 into S. In the absence of a counteracting mechanism(s) by the virus, induction of a p53 response leading either to cell cycle arrest or apoptosis would be expected to block virus growth. Expression of polyomavirus large T in NIH 3T3 cells can overcome p53-dependent arrest by a mechanism dependent on large T interaction with pRb (20, 25). In REF52 established rat fibroblasts, which express normal p53, polyomavirus large T and/or small T can block signaling between p19ARF and p53 (39, 43). Middle T by itself fails to override p53 (19) and also fails to transform REF52 cells unless accompanied by large and/or small T (43). These studies were carried out with subviral constructs or in nonpermissive cells, i.e., under conditions where normal virus replication does not occur. The present study was undertaken to gain a better understanding of the effects of polyomavirus on p53 during lytic infection of mouse cells and of how the virus might override effects of p53.

MATERIALS AND METHODS

Cells and viruses. Primary baby mouse kidney (BMK) cells were prepared from 15-day-old baby mouse kidneys (63). INK-4a−/− mouse embryo fibroblasts (MEF) (third passage) were a generous gift of Ron DePinho and Norman Sharpless of the Dana-Farber Cancer Institute, Boston, Mass. p53−/− MEF were derived from p53-deficient mice from our colony (18) or from James DeCaprio of the Dana-Farber Cancer Institute. Polyomaviruses were the wild-type laboratory strain RA (24), the highly virulent strain LID (4), and the RBl mutant encoding large T defective for binding of pRb (26). Monolayers were maintained in Dulbecco’s minimal essential medium supplemented with 10% fetal bovine serum for BMK and MEF, respectively. Cells were infected at a multiplicity of infection (MOI) of 1 to 30 PFU. The percentage of cells infected was determined by nuclear staining for large T by indirect immunofluorescence, using rat polyclonal anti-T antigen (T Ag) (55) and fluorescein isothiocyanate-conjugated anti-rat immunoglobulin G (IgG) (Jackson ImmunoResearch).

Immunoprecipitation and immunoblotting. Lysates of infected cells were prepared at the indicated times with NP-40 lysis buffer (20 mM Tris, pH 7.5, 135 mM NaCl, 1 mM MgCl2, 0.1 mM CaCl2, 10% glycerol, 1% NP-40, 0.1 mM Na3VO4, 50 mM β-glycerophosphate, 10 mM NaF, and the protease inhibitor Complete Mini from Roche) and were used for immunoblotting. For immunoprecipitations cells were lysed in buffer containing 20 mM Tris, pH 7.5, 135 mM NaCl, 1% glycerol, 1% NP-40, 0.1 mM Na3VO4, 10 mM β-glycerophosphate, 10 mM NaF, and the protease inhibitor Complete Mini from Roche. For immunoblotting, 70 µg of protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel elec-
trophoresis. For immunoprecipitations, extracts (0.5 to 1 mg of protein) were incubated with antibody at 4°C overnight and the immune complexes were recovered with protein A-Sepharose CL-4B (Amersham Pharmacia). The proteins were resolved on 10 or 12.5% polyacrylamide gels and were transferred to nitrocellulose membrane. Antibodies for immunoblotting were rabbit polyclonal anti-p53 (CM5) from Novocasta; monoclonal anti-p53 (Ab-3, Pab 240) from Oncogene Research; rabbit polyclonal anti-p21 (C-19), rabbit polyclonal anti-BAX (N-20), goat polyclonal anti-p19Arf (M-20), rabbit polyclonal anti-mdm2 (H-221), mouse monoclonal ant actin (C-2) (all from Santa Cruz), and rat polyclonal anti-T Ag ascites (55). Antibodies for immunoprecipitation were rabbit polyclonal anti-p21 (C-19) from Santa Cruz, rabbit polyclonal anti-p53 or rabbit polyclonal anti-phosphoserine-15 p53 from Cell Signaling, and monoclonal anti-p53 (Ab-1, Pab 421) from Oncogene Research.

To block immunoprecipitation by anti-phosphoserine-15 p53, 2.5 µl of antibody and 2.5 µl of phosphoserine-15 blocking peptide (Cell Signaling) in 95 µl of lysis buffer were incubated on ice for 2 h before addition of cell extract.

**Immunocytochemistry.** Infected cells were fixed with 3.7% neutral buffered formalin at room temperature for 20 min. After permeabilization with 0.3% Triton X-100 in phosphate-buffered saline (PBS) for 20 min, the cells were blocked with 5% normal donkey serum (Jackson Immunoresearch) in PBS. Cells were stained for T Ag with polyclonal anti-T ags (55), followed by fluorescein isothiocyanate-conjugated anti-rat IgG (Jackson Immunoresearch).

To block immunoprecipitation by anti-phosphoserine-15 p53, 2.5 µl of antibody and 2.5 µl of phosphoserine-15 blocking peptide (Cell Signaling) in 95 µl of lysis buffer were incubated on ice for 2 h before addition of cell extract.

**RESULTS**

Stabilization of p53 and induction of p53 target genes during productive infection by polyomavirus. Extracts of wild-type-polyomavirus-infected BMK cells were prepared at various times after infection, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and blotted with antibodies to p53 and p53 target genes p21Cip1/WAF1 (p21) and BAX (Fig. 1). Levels of p53 in uninfected cultures were low and remained constant over the 40-h time course examined; p21 was barely detectable. In infected cultures, levels of p53 and p21 began to rise around 12 h, coincident with the appearance of large T. By 24 h postinfection, the amounts of p53 and p21 rose 10- to 20-fold or more compared to those in uninfected cultures. The slight declines in infected cultures at the later time points coincide with the development of cytopathic effects and loss of cells from the monolayers. Levels of BAX were low and somewhat variable in uninfected cultures but were seen to rise upon infection in most experiments but to a lesser degree than those of p21. Induction of p53 and its targets following polyomavirus infection resembled that seen in uninfected cells treated with 5 nM actinomycin D (Act D) for 24 h. When a rabbit polyclonal antibody to mdm2 capable of recognizing the 90-kDa form and the p60 cleavage product was used, little or no change in levels was seen in either infected cultures or in Act D-treated cells (not shown). The rise in levels of p53 in infected BMK cells is thus accompanied by induction of some but not all p53-responsive genes. Different patterns of p53 response may occur among the many different cell types that the virus is known to lytically infect in vivo (15).

p21 is known to be induced at the transcriptional level by p53 (23, 38) but may also be regulated through E2F-1 in a p53-independent manner (30). To determine if the increase in p21 in virus-infected cells is p53 dependent, experiments were carried out with p53−/− and p53+/− MEF. Results in Fig. 2 show that induction of p21 following polyomavirus infection is dependent on p53. The induction of p21 by Act D is also p53 dependent in these cells.

The p53 response in polyomavirus-infected cells is accompanied by phosphorylation of p53 on serine 18 and does not depend on the mdm2/p19Arf pathway. Regulation of p53 levels occurs primarily through posttranslational mechanisms (1). One of these depends on mdm2 binding to p53, leading to p53 degradation via ubiquitin-dependent proteolysis (45, 52). This pathway is opposed by the p19Arf product of INK-4a, which binds and sequesters mdm2 in nucleoli leading to accumulation of p53 (62). To determine if p53 accumulation following polyomavirus infection depends on p19Arf, normal and INK-
FIG. 3. Accumulation of p53 in polyomavirus-infected cells does not depend on the p19Arf/mdm2 pathway. Early-passage wild-type MEF and Ink4a−/− MEF were infected as described for Fig. 1A. Extracts were prepared at the times indicated and were analyzed by immunoblotting. p53 was detected with anti-p53 (CM5 from Novocastra).

4a−/− MEF were infected and analyzed. Levels of p53 and p21 were seen to increase in the same manner in both sets of cultures (Fig. 3). These results, as well as the finding of little or no change in levels of mdm2 and p19Arf (not shown), indicate that p53 accumulation in polyomavirus-infected BMK cells is not primarily dependent on p19Arf.

Phosphorylation of p53 occurs at multiple sites in response to various stimuli, and these modifications are important in mediating p53’s stability and functions (1, 5, 21, 33, 37, 40, 52–54). DNA damage from UV or ionizing radiation leads to phosphorylation on serines 18 and 23 of murine p53 involving ATM, ATR, CHK1/2, or other kinases (9, 29, 50, 51, 59). Phosphorylation of p53 at serines 15 and 20 (serines 18 and 23 in mouse p53) is known to prevent the binding of mdm2 to p53 and the ability of mdm2 to inhibit p53 transactivation (12, 52). Mutation of serines 15 and 20 to alanine prevents full stabilization of p53 in vivo (10, 60), and mutation of mouse serine 18 to alanine in murine embryonic stem cells reduces p53 accumulation in response to DNA damage (9). A phosphopeptide antibody specific for p53 phosphorylated on serine 18 was used in an immunoblot of extracts from uninfected, infected, and Act D-treated BMK cells. Polyomavirus infection clearly leads to phosphorylation of p53 on serine 18, as does the DNA damage induced by Act D (Fig. 4).

Induction of a p53 response in vivo. To determine whether lytic infection in the intact host is also accompanied by a p53 response, extracts of kidneys of 8-day-old neonatally infected mice were prepared and analyzed. The virulent LID strain of polyomavirus was used because of its ability to cause rapid and extensive lytic damage in the kidney (4, 6). Extracts of kidneys from LID-infected mice showed significant elevations of p53 compared to extracts from kidneys of uninfected mice (Fig. 5). Stabilization of p53 in the virus-infected kidney is accompanied by induction of p21 and by phosphorylation of p53 on serine 18. The p53 response induced by polyomavirus lytic infection in vitro and in vivo resembles that induced by DNA damage in all respects examined thus far.

Induction of a p53 response does not require entry of cells into S phase and synthesis of viral DNA. In spite of induction of p53 and p21, polyomavirus drives cells into S phase during lytic infection in a manner dependent on large T binding to pRb (20, 25). To determine whether p53 induction by the virus requires override of the G1 block and synthesis of viral as well as cellular DNA, levels of p53 and p21 were compared in cells infected by wild-type virus and the RB1 virus mutant. RB1 encodes a large T defective in binding to pRb, is inefficient in inducing S-phase entry, and shows delayed and reduced levels of viral DNA synthesis (25). BMK cultures were infected by wild-type or mutant virus at MOIs of around 1, leading to roughly two-thirds of the cells becoming T Ag positive in each set of cultures. Cells infected by the wild-type virus progressed into S phase, accompanied by a roughly 18-fold increase in the level of phosphorylated p53 and a 10-fold increase in p21. In contrast, cells infected by RB1 at a matched MOI showed increases of less than twofold in the same parameters (Fig. 6). At considerably higher MOIs, the RB1 mutant was able to induce a larger p53 response. Binding of pRb by large T is therefore essential for efficient induction of a p53 response. These results are consistent with those reported earlier, indicating p21 induction by wild-type large T but not by a pRb binding mutant (49).

To determine the efficiency of induction of a p53 response by wild-type virus at the cellular level and to confirm that the RB1 mutant is impaired in inducing this response, infected BMK cells were examined by double indirect immunofluorescence...
staining using anti-T and anti-phosphoserine-18 p53 antibodies. Images are shown in Fig. 7, and quantitation is given in Table 1. It is clear that a majority of wild-type-virus-infected cells that become T Ag positive at 18 h postinfection also show induction of phosphoserine-18 p53. The percentage of T Ag-positive cells that are also phosphoserine-18 p53 positive rises with time after infection, from 62% at 18 h to 88% at 31 h. With the RB1 mutant at 18 h, only 3.4% of cells are doubly positive. The percentage rises with time but remains well below that achieved by the wild-type virus, consistent with the known defects and leakiness of this mutant (25).

Induction of p53 by the virus may depend on function(s) of one or more E2F family members that are activated following large T:pRb interaction but preceding actual S-phase entry. To test this possibility, BMK cells were infected by wild-type virus allowing release and activation of E2F and were incubated with mimosine to impose a block at the G1/S boundary (35). Under these conditions, p53 and p21 were induced normally, along with phosphorylation of p53 on serine 18 (Fig. 8). Entry of infected cells into S phase and synthesis of viral DNA are therefore not required for induction of a p53 response. Rather, the response depends on large T interaction with pRb and

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<th>TABLE 1. Incidence of T Ag and phosphoserine-18 p53 (Pp53) in wild-type-virus-infected and mutant-RB1-infected BMK cells</th>
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FIG. 7. Immunofluorescence staining of BMK cells infected with wild-type or mutant RB1 virus for T Ag and phosphoserine-18 (Pser18) p53. Confluent BMK cells were infected with wild-type (WT) or RB1 virus at an MOI of 1 or 2 and incubated for 18 h. To block possible nonspecific staining, antibody to phosphoserine-18 p53 was incubated with the antigenic peptide for 1 h before being applied to cells on coverslips. DAPI, 4′,6′-diamidino-2-phenylindole.

FIG. 8. Induction of phosphoserine-18 (Pser18) p53 and p21 occurs in cells blocked in S-phase entry by mimosine. Confluent BMK cells were either uninfected or infected with wild-type polyomavirus at an MOI of 1. At 4 h postinfection mimosine was added to a concentration of 0.2 or 0.5 mM. Cells were harvested at 24 h postinfection and were analyzed by immunoblotting, T Ag staining, and fluorescence-activated cell sorting.
most likely activation of E2F function(s). Cellular genes required for viral DNA synthesis are known to be induced by E2F-1. Beside G1 cyclins, cyclin-dependent kinases, and various enzymes needed for DNA synthesis, these include DNA polymerase α (“primase”) and PCNA (17, 22). The latter assemble along with RPA, topoisomerase I, and large T at the viral replication origin and are essential for the initiation reaction and replication (56).

**Polyomavirus large T coprecipitates with phosphorylated p53 and with p21.** Earlier attempts at demonstrating direct interaction between p53 and polyomavirus large T were negative using a monoclonal antibody that effectively brings down SV40 large T:p53 complexes (61). To determine if polyomavirus might interact with phosphorylated p53, immunoprecipitation of an infected cell extract was carried out using serine 18 phosphopeptide antibody followed by blotting with anti-T. This antibody brought down significantly more large T than either control or other anti-p53 antibodies (Fig. 9A). The ability of the phosphopeptide antibody to coprecipitate large T was effectively inhibited by preincubation with phosphopeptide (Fig. 9B). Polyclonal rabbit anti-p53 gave various but generally low amounts of large T by immunoprecipitation in comparison with anti-phosphoserine-18 p53. Thus, while polyomavirus large T coprecipitates with p53 phosphorylated on serine 18, some form of interaction with unphosphorylated p53 or p53 modified at other sites is also possible. Neither normal IgG nor the mouse monoclonal antibody to p53 previously found to be negative (61) was found to coprecipitate polyomavirus large T.

The E7 protein encoded by the high-risk HPVs, in addition to binding pRb, also interacts with p21. This interaction contributes to the ability to override p53-mediated G1 arrest and to promote continued cell division in differentiating keratinocytes (27, 32). The possibility that a similar interaction occurs between polyomavirus large T and p21 was investigated using anti-p21 as the precipitating antibody and extracts of infected BMK cells. A substantial amount of large T was found to coprecipitate with anti-p21 (Fig. 9C). Thus, large T may act in a manner similar to that of HPV E7 in binding p21 and blocking its cell cycle inhibitory functions.

**DISCUSSION**

Mice developing polyomavirus tumors show no evidence of effects attributable to the virus leading to a p53 block (18). Polyomavirus tumors fail to show stabilization of p53 in the manner of SV40-induced tumors, and the majority of primary polyomavirus tumor-derived cell lines retain p53 and show a normal p53 response to DNA damage (18). Thus, in the context of tumor cell growth, polyomavirus appears neither to elicit nor to block p53.

In contrast, results of the present study clearly show a p53 response elicited by polyomavirus during productive viral infection, both in cell culture and in the mouse. Lytic infection and virus amplification are essential steps leading to the induction of tumors. These findings thus raise questions at two levels. First, what is the mechanism of p53 induction operating in productively infected cells but not in nonproductively infected (i.e., tumor) cells, and second, how is the virus able to circumvent the cell cycle arrest and proapoptotic functions of p53 during its own replication?

In polyomavirus-infected primary BMK cells, p53 begins to accumulate along with expression of the T Ags and prior to the onset of viral DNA replication. By 20 to 24 h postinfection, levels of p53 are 10- to 20-fold higher than those found in mock-infected cells. Coincident with its accumulation, p53 becomes phosphorylated on serine 18, leading to its stabilization. p53 target genes are selectively induced. p21 is highly induced, BAX is induced to a lesser degree, and mdm2 remains essentially unchanged. This pattern of induction is similar to that seen in the same cells following Act D treatment. This suggests that lytic infection by polyomavirus in some manner triggers a DNA damage-like response. The latter typically involves activation of ATM, ATR, DNA-PK, and the CHK1 and CHK2 kinases, which are known to phosphorylate p53 on N-terminal serine residues. Though the specific pathway leading to p53 phosphorylation has not been determined, some aspect of this pathway upstream of p53, or possibly the p53 response itself, may be important to the virus.

Results suggest that the viral trigger for induction of a p53 response in a lytic infection may be viral DNA replication initiation complexes. These complexes, best characterized for
SV40 (56), consist of large T bound to the viral origin along with replication proteins encoded by the host. The viral DNA is partially unwound at the origin, mimicking cellular DNA origins that have fired (“theta” structures). Results from two experiments support this interpretation and at the same time indicate an important role for E2F-1. The large T mutant RB1, which is defective in binding pRb and in inducing cellular as well as viral DNA synthesis, is also defective in inducing a p53 response. Wild-type virus, on the other hand, is able to induce p53, even when blocked from inducing synthesis of viral and cellular DNA by addition of mimosine to infected cultures. Under these conditions, wild-type large T is expected to bind pRb leading to activation of E2F-1-responsive genes. The latter include DNA polymerase α and PCNA (17, 22), which function along with T antigen in viral DNA initiation and replication complexes (56). Thus, cells harboring free viral DNA with fired origins but unable to enter S phase may respond with a DNA damage response.

Two other mechanisms of induction are possible but less likely. One is that the introduction of linear fragments of cellular DNA via pseudovirions (42) would mimic the generation of double-strand breaks by ionizing radiation and thereby induce a p53 response. This seems unlikely to be the only mechanism, since the RB1 mutant is impaired in its ability to induce the response. A second possibility is that induction of p19Arf by E2F-1 (3) would lead to p53 stabilization by interference with mdm2-dependent p53 degradation (62). In an established line of rat fibroblasts, middle T can induce a p53-dependent cell cycle block via p19Arf, a block that large and/or small T can overcome (39). In the mouse system, however, stabilization of p53 occurs following polyomavirus infection of INK-4a−/− MEFs and is therefore not dependent solely on the p19Arf/mdm2 pathway.

Induction of p53 during lytic infection potentially could lead to G1 arrest or to apoptosis. Either cellular response would effectively block virus replication. However, several mechanisms are available to the virus to bypass or block p53 responses. First, the binding of pRb by large T would be expected to act downstream of p21, bypassing the G1 arrest brought about by inhibition of the G1 cyclin–cyclin-dependent kinases acting on pRb. Induction of p21, which can be brought about by the virus either via p53 or possibly E2F-1 (30), may conceivably serve a positive function for the virus by titrating the levels of free p21 so as to aid in the assembly and promote rather than inhibit the activity of these kinases (30, 36, 64).

Coprecipitation of large T with phosphorylated p53 may indicate direct or indirect association. Only a small fraction of large T appears to be involved. Interaction could be mediated by the CBP/p300 coactivators of p53 which also bind polyomavirus large T (11, 43a). Alternatively, large T and p53 may be brought together on the viral DNA, which contains p53 and large T binding sites adjacent to the core origin (42a). The anti-p53 monoclonal antibody Pab 421 fails to bring down complexes with polyomavirus large T (reference 61 and present results). This antibody binds to p53 at the C terminus (57) and effectively brings down complexes with SV40 large T, which binds to the central DNA-binding domain of p53 (2, 58). This suggests that polyomavirus large T may interact differentially from SV40 large T by binding at the C terminus of p53 such that these complexes would not be recognized by the Pab 421 monoclonal antibody. Large T does not block the induction of p21 by p53. However, the binding of large T to p21 provides a plausible mechanism for countering the cell growth arrest functions of p53 in a manner similar to that shown for HPV E7 (27, 32).

The proapoptotic functions of p53 mediated by BAX could well be overridden by middle T activation of the phosphatidylinositol 3-kinase/Akt pathway, which is known to protect against apoptosis in virus-infected cells (13, 41, 46). Akt can block apoptosis at several levels, including phosphorylation of BAD, caspases, and p21 (7, 14, 65). Both the hamster and mouse polyomaviruses encode middle T proteins that activate this antiapoptotic pathway (13, 48), distinguishing them from other DNA tumor viruses that lack a middle T and that target p53 more directly.

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