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p53-dependent G_1 arrest involves pRB-related proteins and is disrupted by the human papillomavirus 16 E7 oncoprotein

(*CIP1/WAF1*/cervical carcinoma/DNA damage/carcinogenesis)

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ABSTRACT **The cell cycle regulatory tumor suppressor proteins p53 and pRB are targeted for inactivation by several tumor viruses, including the high-risk types of human papillomaviruses (HPVs) via interactions of the HPV E6 and E7 oncoproteins with p53 and pRB, respectively. p53 plays a central role in a signal transduction pathway that mediates G_1 arrest after DNA damage, though the mechanism by which G_1 arrest occurs has not been elucidated. The cyclin-associated protein p21$^{waf1/cip1}$ has recently been shown to be induced by p53 and to inhibit cyclin complex-mediated phosphorylation of pRB *in vitro*. Thus, we investigated a possible role for pRB in the p53-mediated DNA damage response. After γ-irradiation, cells expressing wild-type p53 arrested in G_1, contained increased levels of *WAF1/CIP1* mRNA, and demonstrated accumulation of hypophosphorylated pRB. In contrast, cell lines with abnormal p53 genes or with p53 functionally inactivated by the E6 oncoprotein of HPV16 (a high-risk HPV) failed to arrest in G_1, did not elevate *WAF1/CIP1* mRNA, and did not accumulate hypophosphorylated pRB. Despite apparently normal elevation of p53 protein and *WAF1/CIP1* mRNA after irradiation, cells expressing HPV16 E7 also failed to arrest in G_1 and did not accumulate hypophosphorylated pRB. Disruption of *RB* genes alone did not totally abrogate this G_1 arrest. Our results suggest that p53 indirectly regulates phosphorylation of pRB and that pRB and/or other pRB-like molecules that bind to HPV16 E7 participate in the DNA damage-mediated G_1 arrest signal. In the process of HPV infection, the HPV E6 and E7 oncoproteins may undermine this cell cycle checkpoint, contributing to the accumulation of genetic alterations during tumorigenesis.**

The tumor suppressor genes p53 and pRB are inactivated during the development of a wide variety of human cancers. Inactivation of both p53 and pRB can occur via genetic mechanisms or through interaction of their gene products with cellular or viral proteins. High-risk human papillomaviruses (HPVs) presumably contribute to the development of HPV-associated cancers through the interaction of the HPV E6 oncoprotein with p53 and the HPV E7 oncoprotein with pRB and related proteins such as p107 (for review, see ref. 1). The functional consequences of these protein–protein interactions remain poorly understood.

We have previously shown that arrest in the G_1 phase of the cell cycle after sublethal DNA damage is mediated by wild-type p53 (wtp53) (2–4) and that expression of the E6 oncoprotein encoded by high-risk HPVs (such as HPV16) abrogates the G_1 arrest (5). Loss of p53 function is associated with certain types of genetic instability, such as gene amplification and aneuploidy (6–8). A role for pRB in this cell cycle checkpoint pathway has not been established. Recently, a relationship between p53 and pRB in cell cycle regulation was suggested by the cloning and characterization of the *WAF1/CIP1* gene on chromosome 6p [also referred to as *SDI1* for senescent cell-derived inhibitor (9) and *PIC1* for p53-regulated inhibitor of cdks (10)]. The *CIP1* gene was identified on the basis of the interaction between cip1 and cdk2 (11). The identical gene, *WAF1*, was independently cloned on the basis of its inducibility by p53 (12). The product of this gene, p21$^{waf1/cip1}$, had been reported previously on the basis of its interaction with several cyclin–cdk complexes (13). The p21$^{waf1/cip1}$ protein is a potent growth suppressor (12) and may be one of the major effector molecules of p53. It is also a potent inhibitor of cell cycle-regulated kinases that target pRB and pRB-related molecules (11), at least *in vitro*. These discoveries suggested a potential link between p53 and pRB in tumor suppression and cell cycle regulation and raised suspicion that pRB could be a functional participant in the p53-dependent DNA damage response pathway.

The waf1/cip1 protein may participate in cell cycle regulation through the inhibition of cyclin–cdk complex-mediated phosphorylation of pRB (11). The E2F family of transcription factors interact with pRB and related molecules such as p130 and p107 (for review, see ref. 14). During most of the G_1 phase of the cell cycle, E2F is found in complexes with the hypophosphorylated form of pRB. In late G_1, the majority of pRB becomes hyperphosphorylated, thereby releasing transcriptionally active E2F. Unbound E2F transcription factors stimulate transcription of cellular genes involved in growth control and DNA synthesis. Through this cell cycle-dependent phosphorylation, pRB can either inhibit or enhance E2F-dependent transcription, an effect that correlates with the growth suppressor function of pRB (15, 16). The interaction of the HPV E7 oncoprotein with pRB has essentially the same effect as pRB phosphorylation [i.e., increased E2F transcriptional activity (17)].

In this study, we investigated the relationship between pRB and p53 in the p53-mediated cell cycle checkpoint pathway. We demonstrate that the G_1 cell cycle arrest occurs prior to pRB phosphorylation and that accumulation of hypophosphorylated pRB in response to DNA damage requires elevation of wtp53 protein. Furthermore, we show that the G_1 cell cycle arrest can be abrogated not only by HPV16 E6 through its interaction with p53 but also by HPV16 E7 through its interaction with pRB and perhaps through pRB-related molecules. Finally, pRB and/or pRB-related proteins appear to be checkpoint participants downstream of p53 and p21$^{waf1/cip1}$ in the p53-mediated response to DNA damage. Thus, high-risk HPVs encode proteins capable of disrupting this important cellular response at two distinct points. Through this mechanism, HPV E6 and E7 oncoprotein ex-

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Abbreviations: HPV, human papillomavirus; wtp53, wild-type p53.
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pression may contribute to the accumulation of genetic alterations responsible for cervical and other HPV-associated cancers.

MATERIALS AND METHODS

Cell Lines and Plasmids. The source and culture conditions for primary keratinocytes (5), mouse embryo fibroblasts (4, 6, 18), and cell lines RKO (wtp53), ML-1 (wtp53), HL-60 (p53 null), and SW480 (mutant p53) (2, 3) have been reported. The HPV16 E7 expression plasmid was obtained by cloning the full-length E7 open reading frame (map positions 544–875), from pJ6Ω-16E7 (provided by Attila Lorincz, Digene Diagnostics, Silver Spring, MD), into the unique *Bam*HI cloning site of the pCMVneo expression vector (19) downstream of the cytomegalovirus promoter. The DNA insert was sequence verified in its entirety. Logarithmically growing RKO cells were transfected with either pCMVneo-16E7 or pCMVneo vector without insert by using Lipofectin as described by the manufacturer (GIBCO/BRL). Transfected cells were initially selected in G418 (GIBCO/BRL) at 1.0 mg/ml, and after 10 days single colonies were cloned by limiting dilution in G418 at 0.5 mg/ml. Two independent E7-expressing cell lines (RC07-6 and RC07-14), as determined by Southern blot analysis, were chosen for further analysis. Both lines were shown to express E7 protein by immunoprecipitation (data not shown). RKO clonal cell lines expressing HPV16 E6 (RC10-1, RC10-2, and RC10-3) have been described (5).

p53 and pRB Protein Detection by Immunoblotting. Levels of p53 protein were measured 4 hr after cells were exposed to 0 or 4 grays (Gy) of γ-irradiation in a ^{137}Cs irradiator at 1 Gy/min as described (5). The cells were lysed directly in SDS/PAGE loading buffer, and total protein was quantified using an amido blue black assay. Total cellular protein (100 μg) was separated on an SDS/8% PAGE gel and transferred to nitrocellulose (Hybond-ECL; Amersham) using a semidry electroblotter (Millipore). p53 protein was detected after incubation with 1 μg of monoclonal antibody DO-1 (Oncogene Science), and the bound antibody was visualized with enhanced chemiluminescence (ECL; Amersham).

pRB was evaluated by immunoblot at 1, 3, and 24 hr postirradiation with 4 Gy and compared to pRB in untreated cells. For this analysis, the samples were separated by SDS/6% PAGE, and pRB was detected with 1 μg of monoclonal antibody PMG3-245 (PharMingen) as described above for p53.

***WAF1/CIP1* Expression in RKO Cell Lines.** For the detection of *WAF1/CIP1* expression, cells were grown to 80% confluency, and total cellular RNA was isolated as described (20). Irradiated cells were harvested 4–6 hr after irradiation. Twenty micrograms of total RNA was size fractionated on 1.5% agarose-Mops/formaldehyde gels, transferred to nylon membranes, and hybridized to a full-length *WAF1/CIP1* cDNA probe (12) (provided by B. Vogelstein, The Johns Hopkins Oncology Center) in rapid hybridization buffer (Amersham). The blots were washed at 65°C in 0.3× SSC/0.1% SDS and then autoradiographed. The same blots were hybridized to a glyceraldehyde-3-phosphate dehydrogenase cDNA probe to control for potential differences in loading from sample to sample.

Cell Cycle Analysis. To analyze perturbations of cell cycle progression after DNA damage, cells were grown to 80% confluency and treated with 0 or 4 Gy of γ-irradiation. Cell cycle status was determined by dual-parameter flow cytometric analysis of cells labeled with 10 μM BrdUrd for 4 hr at 17 hr postirradiation. The 17-hr time point has been previously shown to accurately reflect p53-dependent cell cycle arrest in several cell lines (2–4). Murine fibroblasts were assayed at either 17 or 24 hr postirradiation. BrdUrd-labeled cells were subsequently stained with a fluorescein isothiocyanate-conjugated anti-BrdUrd antibody (Becton Dickinson) and, for total DNA content, with propidium iodide, as described (2).

RESULTS

Phosphorylation of pRB Is Associated with Functional Activity of p53. Immunodetection of pRB in whole cell lysates shows a complex pattern of protein species, including the more rapidly migrating hypophosphorylated form (pRB110) and a number of more slowly migrating hyperphosphorylated forms (ppRb112–120) (21), which can be distinguished by SDS/PAGE. To investigate the phosphorylation status of pRB in relation to functional p53 activity, we initially chose three cell lines, ML-1 (wtp53), HL60 (p53 null), and SW480 (mutant p53). After γ-irradiation, ML-1 cells show elevated wtp53 protein levels and arrest in G_1, whereas HL60 and SW480 cells do not (2–4). The p53-independent G_2 arrest in response to γ-irradiation remains intact in each of these cell lines (2, 3). pRB phosphorylation in all three cell lines was determined by immunoblot analysis at 1, 3, and 24 hr after irradiation with 4 Gy and compared to unirradiated cells (Fig. 1). In ML-1, increased hypophosphorylated pRB was seen at 3 hr after γ-irradiation and was still present 24 hr postirradiation. The levels of hypophosphorylated pRB remained constant in HL60, and this form of pRB was undetectable in SW480 before or after irradiation. These results suggest a relationship between the functional activity of p53 and pRB phosphorylation in DNA damage-induced G_1 arrest and indicate that the arrest occurs prior to pRB phosphorylation. However, these findings do not address whether accumulation of hypophosphorylated pRB is a cause or a consequence of G_1 arrest; this distinction would require evaluation of the DNA damage-induced G_1 arrest after abrogation of pRB function.

pRB Phosphorylation in HPV E6- and E7-Transfected RKO Lines. The association between p53 accumulation after DNA damage and the appearance of hypophosphorylated pRB was also tested in RKO cells (Fig. 2). After γ-irradiation, these cells also show elevated wtp53 protein and arrest in G_1 (3, 5). In untransfected RKO cells (Fig. 2*A*) or RKO transfected with vector alone (data not shown), increased hypophosphorylated pRB was detectable 3 hr after treatment. In two RKO cell lines in which p53 is functionally inactivated by HPV16 E6 and that fail to arrest in G_1 following DNA damage (5), no significant change in hypophosphorylated pRB was identified following DNA damage (results obtained with one representative line, RC10-2, are shown in Fig. 2*A*). These results

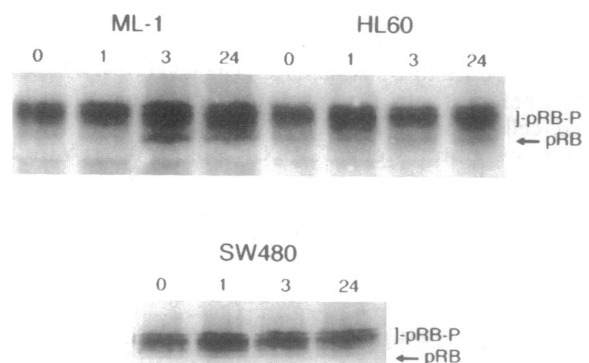

FIG. 1. Phosphorylation status of pRB by immunoblot in cell lines ML-1, HL60, and SW480 after DNA damage. pRB was detected in cellular lysates from unirradiated cells (0 hr) and in cells 1, 3, and 24 hr after irradiation with 4 Gy. Lanes 1–4, cell line ML-1 (wtp53 and pRB); lanes 5–8, cell line HL60 (p53 null and wild-type RB); lanes 9–12, cell line SW480 (mutant p53 and wild-type RB).

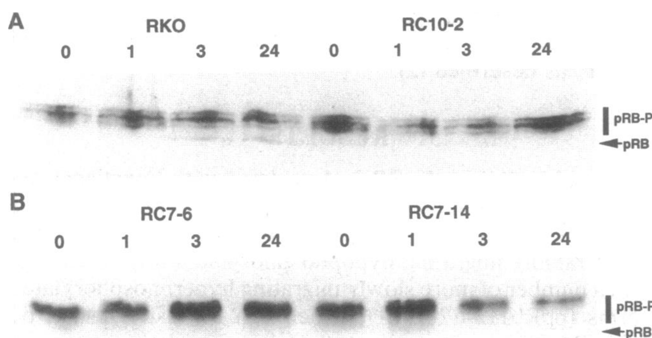

FIG. 2. Phosphorylation status of pRB by immunoblot in RKO-derived cell lines. pRB was detected in cellular lysates from unirradiated cells (0 hr) and in cells 1, 3, and 24 hr after irradiation with 4 Gy. (*A*) pRB in untransfected RKO cells (lanes 1–4) and in RC10-2, which expresses HPV16 E6 (lanes 5–8). (*B*) pRB in two independent HPV16 E7-transfected RKO lines, RC7-6 and RC7-14.

provide further evidence that accumulation of wtp53 plays an important role in the regulation of pRB phosphorylation during the DNA damage response. RC07-6 and RC07-14 express both HPV16 E7 mRNA and protein (data not shown). We also analyzed the phosphorylation status of pRB in RC07-6 and RC07-14 (Fig. 2*B*). In the two HPV16 E7-expressing RKO cell lines there is no accumulation of the hypophosphorylated form of pRB after DNA damage.

p53 Accumulation After DNA Damage in HPV16 E6- and E7-Transfected RKO Cells. To investigate whether RKO cells expressing HPV16 E7 retain the ability to elevate p53 protein levels in response to DNA damage, we treated the lines with 0 or 4 Gy of γ-irradiation and assayed the levels of p53 protein 4 hr after treatment. As shown in a previous study (5), untransfected RKO cells show increased p53 protein levels after irradiation, whereas p53 is essentially undetectable in RC10-1, the HPV16 E6-transfected RKO line (Fig. 3). The E7 transfectants, however, show elevation of p53 after irradiation comparable to untransfected RKO cells, indicating that HPV16 E7 expression does not interfere with events that trigger the accumulation of p53 in response to DNA damage in these cells.

Expression of *WAF1/CIP1* in RKO Cells. The expression of *WAF1/CIP1*, a recently identified gene, is induced by p53 (12). We hypothesized that *WAF1/CIP1* induction may be part of the p53-mediated DNA damage response. RNA blot analysis was used to determine the expression of *WAF1/CIP1* in normal cervical keratinocytes (PCx22), RKO cells transfected with vector alone (RCneo-1), and in HPV16 E6 (RC10-1)- and E7 (RC07-6 and RC07-14)-transfected RKO cells in response to DNA damage. The level of *WAF1/CIP1* mRNA before irradiation is approximately equivalent in all tested cells. Four to 6 hr after treatment with 4 Gy of irradiation, untransfected RKO cells and HPV16 E7-expressing cells demonstrate a severalfold increase in *WAF1/CIP1* mRNA levels. In contrast, the level of *WAF1/CIP1* mRNA in HPV16 E6-expressing RKO cells is unchanged after irradiation (Fig. 4). Thus, *WAF1/CIP1* induction after DNA damage correlates with p53 accumulation in all of the tested RKO transfectants.

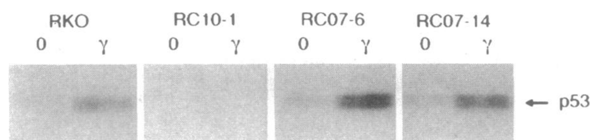

FIG. 3. Detection of steady-state p53 protein levels by immunoblot. Each pair of lanes represents unirradiated cells (0) or cells irradiated with 4 Gy (γ). Lanes 1 and 2, untransfected RKO cells; lanes 3 and 4, RC10-1 (HPV16 E6); lanes 5 and 6, RC07-6 (HPV16 E7); lanes 7 and 8, RC07-14 (HPV16 E7).

Cell Cycle Progression of HPV16 E6- and E7-Transfected RKO Cells After Irradiation. Our results suggest that the hypophosphorylated form of pRB is potentially an important effector "downstream" of p53 in DNA damage-induced G_1 arrest. Because E7 proteins of high-risk HPVs disrupt the function of pRB and possibly other proteins that are important to the regulation of cell cycle progression, we analyzed RC07-6 and RC07-14 for cell cycle arrest after sublethal doses of γ-irradiation. The relative decrease in the S-phase fraction after irradiation in RKO cells expressing E7 was significantly less than in control cells (Fig. 5). Elimination of p53 function by HPV16 E6 similarly eliminated this arrest.

Cell Cycle Changes After Irradiation in Embryonic Fibroblasts Lacking *RB* Genes. Since HPV16 E7 is known to interact not only with pRB but also with pRB-like proteins such as p107 and p130, a role for pRB alone in this pathway was evaluated in cells with homozygously deleted *RB* genes (in which the function of pRB-like proteins remains intact). Embryonic fibroblasts with wild-type *RB* and p53 genes consistently arrested in G_1 after 4 Gy of γ-irradiation, while embryonic fibroblasts with homozygously deleted p53 genes consistently failed to arrest (Fig. 5). Disruption of both *RB* genes in the embryonic fibroblasts did not totally eliminate the G_1 arrest. Since cells expressing HPV16 E7 totally lost the arrest, these findings suggest that pRB-like proteins that interact with E7 (e.g., p107 and/or p130) may, in addition to pRB, act as downstream effectors of the G_1 arrest in response to DNA damage.

DISCUSSION

Cells with an intact DNA damage response accumulate p53 and arrest in G_1 after damage by ionizing radiation or other damaging agents that, like ionizing radiation, cause DNA strand breaks (2, 22, 23). In some situations, this p53-dependent signal appears to lead to apoptotic cell death rather than to G_1 arrest (24–26). Cells that are defective in this pathway fail to arrest in G_1 or fail to undergo apoptosis after DNA damage. Possibly, failure to reach either of these endpoints could lead to the fixation of mutations in daughter cells and thereby contribute to the accumulation of genetic alterations found in human tumors. Previous studies indicate that p53 has a central role in this pathway. However, it seems likely that other molecules are also involved.

wtp53 has recently been found to induce expression of $p21^{waf1/cip1}$ (12), which inhibits the activity of cyclin-associated kinases that normally phosphorylate pRB (11), at least *in vitro*. However, the possible roles of $p21^{waf1/cip1}$ and pRB in the DNA damage response have not been previously investigated. We hypothesized that cells with mutant or

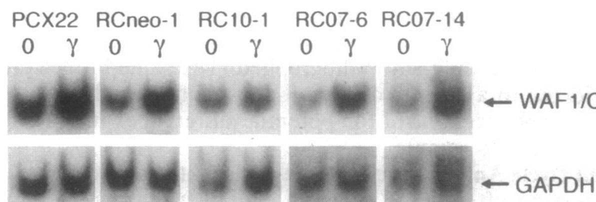

FIG. 4. Expression of *WAF1/CIP1* and glyceraldehyde-3-phosphate dehydrogenase mRNA in RKO cell lines 4–6 hr after irradiation. Each pair of lanes represents unirradiated cells (0) or cells irradiated with 4 Gy (γ). Lanes 1 and 2, normal cervical keratinocytes (PCx22); lanes 3 and 4, RKO cells transfected with vector alone (RCneo-1); lanes 5 and 6, RKO cells transfected with HPV16 E6 (RC10-1); lanes 5–8, RKO cells transfected with HPV16 E7 (RC07-6 and RC07-14).

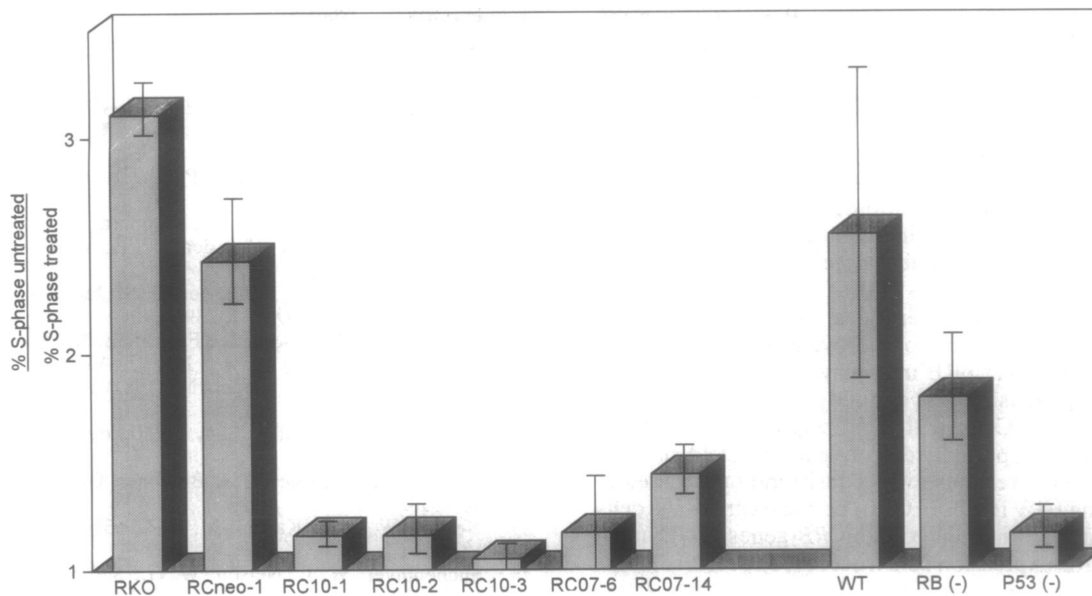

FIG. 5. Cell cycle changes in cells with altered pRB or p53 function after exposure to γ-irradiation. Cell cycle analysis of unirradiated cells or cells after irradiation was performed as described in *Materials and Methods*. The data is plotted as the ratio of the percentage of cells in S phase prior to irradiation to the percentage of cells in S phase after irradiation; mean values of multiple determinations and standard errors are shown. Ratios for control RKO cells [RKO (n = 2), RCneo-1 (n = 5)], RKO cells transfected with HPV16 E6 [RC10-1 (n = 4), RC10-2 (n = 2), RC10-3 (n = 3)], and RKO cells transfected with HPV16 E7 [RC07-6 (n = 3), RC07-14 (n = 3)] are shown. Embryonic fibroblasts from normal mice [WT (n = 3)], mice with disrupted *RB* genes [RB(−) (n = 3)], and mice with disrupted p53 genes [p53(−) (n = 2)] were similarly evaluated.

absent p53 should fail to activate *WAF1/CIP1* in response to DNA damage. In the absence of $p21^{waf1/cip1}$ inhibition of cyclin-dependent kinase activity, substrates such as pRB should be disproportionately phosphorylated. In this scenario, phosphorylated pRB-like proteins would release E2F transcriptional activity, which in turn would activate genes required for entry into the S phase of the cell cycle. A proposed pathway is summarized in Fig. 6.

Regulation of E2F levels during G_1 might also be dependent on p107 and/or p130, pRB-related proteins that also interact with E2F in a cell cycle-dependent manner (18, 28, 29). Like pRB, p107 can inhibit cell proliferation and E2F-mediated transcription (30, 31), although the timing of some p107–E2F interactions may be different from that of pRB (32). While most pRB–E2F complexes are found in G_1, p107 and E2F can be demonstrated in two complexes: one with cyclin A/cdk2, present predominantly in S phase, and another with cyclin E/cdk2 found in G_1 (33).

HPVs are among several DNA tumor viruses that encode oncoproteins that interact with p53 and pRB. The E6 proteins encoded by high-risk HPVs not only bind but also degrade p53 via a ubiquitin-mediated mechanism (34, 35). HPV16 E7 is capable of binding pRB (36) and dissociating pRB–E2F complexes (17, 37, 38). The E7 oncoprotein of high-risk HPVs has also been shown to disrupt the DNA-binding properties of pRB (39). Furthermore, HPV16 E7 has been shown to bind p107 (40) and p130 (41).

In this study we investigated the possible relationship between p53 and pRB in the DNA damage response pathway. RKO and ML-1 cells, which express wtp53, induced *WAF1/CIP1* expression, accumulated hypophosphorylated pRB, and arrested in G_1 after low-dose irradiation. Cell lines with mutant (SW480), absent (HL60), or functionally inactivated (RC10-1 and RC10-2) p53 genes did not induce *WAF1/CIP1*, did not accumulate hypophosphorylated pRB, and did not arrest in G_1 after DNA damage. Thus, we demonstrated that there is a relationship between the functional status of p53 and pRB phosphorylation after DNA damage and that both p53 and pRB have an effect on the regulation of entry into S phase in response to DNA damage.

RKO cells expressing HPV16 E7, which retained induction of p53 and *WAF1/CIP1* following irradiation, failed to accumulate hypophosphorylated pRB or arrest in G_1. These observations suggest that pRB (modulated by phosphoryla-

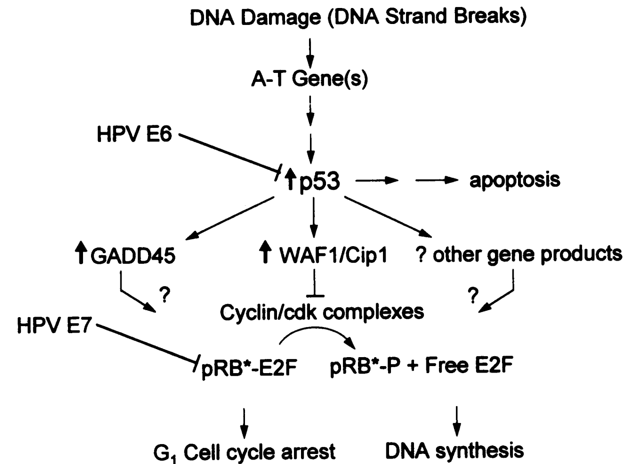

FIG. 6. A proposed representation of the DNA damage-induced cell cycle checkpoint pathway in mammalian cells. DNA damage results in accumulation of p53 protein. Optimal induction of p53 appears to require normal function of the gene products defective in the disease ataxia-telangiectasia after certain types of DNA damage, such as γ-irradiation (4, 27). Accumulation of wtp53 increases levels of *WAF1/CIP1*, which in turn inhibits activation of cyclin–cdk complexes, preventing phosphorylation of pRB-like molecules (pRB*, which includes pRB, p107 and p130). E2F and E2F-like transcription factors therefore remain associated with hypophosphorylated pRB and pRB-like proteins. Bound E2F transcription factors are unable to activate transcription of genes required for progression from G_1 into S phase and, consequently, cells arrest in G_1. High-risk HPVs can disrupt the pathway at two separate points: through interaction of E6 with p53 and, further downstream, through interactions of E7 with pRB or pRB-like proteins. Apoptotic cell death is another response to DNA damage. However, the signals contributing to apoptosis vs. G_1 arrest have yet to be determined. The role of *GADD45* or other gene products in the DNA damage response is not yet clear.

tion) functions downstream of p53 and *WAF1/CIP1* in the control of cell cycle progression. Further evidence that pRB is downstream of p53 is provided by recent studies of immortalized rat embryo fibroblasts transfected with a temperature-sensitive mutant p53 (42). In this system, E7 can overcome growth arrest induced by shifting the cells to the permissive (wtp53 conformation) temperature, with no change in p53 protein levels. In other studies, organotypic culture of HPV16 E7 expressing keratinocytes showed E7-induced proliferation of suprabasal cells that was independent of steady-state p53 levels (43).

The ultimate result of alterations in the p53-mediated growth arrest pathway is loss of cell cycle control. Although our results suggest that pRB may play an important role in this response, pRB is almost certainly not the only downstream participant. Cells with homozygously deleted *RB* genes were, at most, partially defective in this DNA damage response, while the response was consistently abrogated in cells expressing HPV16 E7. In C33A cervical carcinoma cells [which contain both mutant p53 and *RB* genes (44)], introduction of a pRB expression construct failed to restore arrest in G_1, whereas introduction of p107 did cause such an arrest (30). Furthermore, overexpression of wtp53 in Saos-2 cells (p53 null and RB null) results in G_1 arrest (45), suggesting that other proteins besides pRB can act downstream of p53 to effect G_1 arrest. Although the status of p130 in Saos-2 is unknown, p107 is intact in these cells (M. Ewen, personal communication). Taken together, these findings suggest that inactivation of pRB-related proteins is critical for G_1 cell cycle arrest after DNA damage. The study of pRB and other downstream targets in the p53 pathway may eventually improve our understanding of how loss of cell cycle control contributes to neoplastic development and genetic instability.

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1. Vousden, K. H. (1993) *FASEB J.* **7,** 872–879.
2. Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B. & Craig, R. W. (1991) *Cancer Res.* **51,** 6304–6311.
3. Kuerbitz, S. J., Plunkett, B. S., Walsh, W. V. & Kastan, M. B. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 7491–7495.
4. Kastan, M. B., Zhan, Q., El-Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., Plunkett, B. S., Vogelstein, B. & Fornace, A. J., Jr. (1992) *Cell* **71,** 587–597.
5. Kessis, T. D., Slebos, R. J., Nelson, W. G., Kastan, M. B., Plunkett, B. S., Han, S. M., Lorincz, A. T., Hedrick, L. & Cho, K. R. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 3988–3992.
6. Livingstone, L. R., White, A., Sprouse, J., Livanos, E., Jacks, T. & Tlsty, T. D. (1992) *Cell* **70,** 923–934.
7. Yin, Y., Tainsky, M. A., Bischoff, F. Z., Strong, L. C. & Wahl, G. M. (1992) *Cell* **70,** 937–948.
8. Carder, P., Wyllie, A. H., Purdie, C. A., Morris, R. G., White, S., Piris, J. & Bird, C. C. (1993) *Oncogene* **8,** 1397–1401.
9. Noda, A., Ning, Y., Venable, S. F., Pereira-Smith, O. M. & Smith, J. R. (1994) *Exp. Cell Res.* **211,** 90–98.
10. Hunter, T. (1993) *Cell* **75,** 839–841.
11. Harper, J. W., Adami, G. R., Wei, N., Kayomarsi, K. & Elledge, S. J. (1993) *Cell* **75,** 805–816.
12. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, E., Kinzler, K. W. & Vogelstein, B. (1993) *Cell* **75,** 817–825.
13. Xiong, Y., Zhang, H. & Beach, D. (1993) *Genes Dev.* **7,** 1572–1583.
14. Nevins, J. R. (1992) *Science* **258,** 424–429.
15. Weintraub, S. J., Prater, C. A. & Dean, D. C. (1992) *Nature (London)* **358,** 259–261.
16. Arroyo, M. & Raychaudhuri, P. (1992) *Nucleic Acids Res.* **20,** 5947–5954.
17. Morris, J. D. H., Crook, T., Bandara, L. R., Davies, R., Lathangue, N. B. & Vousden, K. H. (1993) *Oncogene* **8,** 893–898.
18. Cobrinik, D., Whyte, P., Peeper, D. S., Jacks, T. & Weinberg, R. A. (1993) *Genes Dev.* **7,** 2392–2404.
19. Baker, S. J., Markowitz, S., Fearon, E. R., Willson, J. K. & Vogelstein, B. (1990) *Science* **249,** 912–915.
20. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
21. Buchkovich, K., Duffy, L. A. & Harlow, E. (1989) *Cell* **58,** 1097–1105.
22. Fritsche, M., Haessler, C. & Brandner, G. (1993) *Oncogene* **8,** 307–318.
23. Nelson, W. G. & Kastan, M. B. (1993) *Mol. Cell. Biol.* **14,** 1815–1823.
24. Slichenmyer, W. J., Nelson, W. G., Slebos, R. J. & Kastan, M. B. (1993) *Cancer Res.* **53,** 4164–4168.
25. Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A. & Jacks, T. (1993) *Nature (London)* **362,** 847–849.
26. Clarke, A. R., Purdie, C. A., Harrison, D. J., Morris, R. G., Bird, C. C., Hooper, M. L. & Wyllie, A. H. (1993) *Nature (London)* **362,** 849–852.
27. Khanna, K. K. & Lavin, M. F. (1993) *Oncogene* **8,** 3307–3312.
28. Shirodkar, S., Ewen, M., Decaprio, J. A., Morgan, J., Livingston, D. M. & Chittenden, T. (1992) *Cell* **68,** 157–166.
29. Cao, L., Faha, B., Dembski, M., Tsai, L. H., Harlow, E. & Dyson, N. (1992) *Nature (London)* **355,** 176–179.
30. Zhu, L., van den Heuvel, S., Helin, K., Fattaey, A., Ewen, M., Livingston, D., Dyson, N. & Harlow, E. (1993) *Genes Dev.* **7,** 1111–1125.
31. Zamanian, M. & La Thangue, N. B. (1993) *Mol. Biol. Cell* **4,** 389–396.
32. Schwarz, J. K., Devoto, S. H., Smith, E. J., Chellappan, S. P., Jakoi, L. & Nevins, J. R. (1993) *EMBO J.* **12,** 1013–1020.
33. Lees, E., Faha, B., Dulic, V., Reed, S. I. & Harlow, E. (1992) *Genes Dev.* **6,** 1874–1885.
34. Scheffner, M., Werness, B. A., Huibregtse, J. M., Levine, A. J. & Howley, P. M. (1990) *Cell* **63,** 1129–1136.
35. Werness, B. A., Levine, A. J. & Howley, P. M. (1990) *Science* **248,** 76–79.
36. Dyson, N., Howley, P. M., Münger, K. & Harlow, E. (1989) *Science* **243,** 934–937.
37. Chellappan, S., Kraus, V. B., Kroger, B., Münger, K., Howley, P. M., Phelps, W. C. & Nevins, J. R. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 4549–4553.
38. Pagano, M., Dürst, M., Joswig, S., Draetta, G. & Jansen-Durr, P. (1992) *Oncogene* **7,** 1681–1686.
39. Stirdivant, S. M., Huber, H. E., Patrick, D. R., Defeo-Jones, D., McAvoy, E. M., Garsky, V. M., Oliff, A. & Heimbrook, D. C. (1992) *Mol. Cell. Biol.* **12,** 1905–1914.
40. Davies, R. C., Hicks, R., Crook, T., Morris, J. D. H. & Vousden, K. H. (1993) *J. Virol.* **67,** 2521–2528.
41. Dyson, N., Guida, P., Münger, K. & Harlow, E. (1992) *J. Virol.* **66,** 6893–6902.
42. Vousden, K. H., Vojtesek, B., Fisher, C. & Lane, D. (1993) *Oncogene* **8,** 1697–1702.
43. Blanton, R. A., Coltrera, M. D., Gown, A. M., Halbert, C. L. & McDougall, J. K. (1992) *Cell Growth Differ.* **3,** 791–802.
44. Scheffner, M., Münger, K., Byrne, J. C. & Howley, P. M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5523–5527.
45. Diller, L., Kassel, J., Nelson, C. E., Gryka, M. A., Litwak, G., Gebhardt, M., Bressac, B., Ozturk, M., Baker, S. J., Vogelstein, B. & Friend, S. H. (1990) *Mol. Cell. Biol.* **10,** 5772–5781.