JOURNAL OF VIROLOGY, Apr. 1997, p. 2905–2912 Vol. 71, No. 4
0022-538X/97/$04.00+0

Analysis of the p53-Mediated G_1 Growth Arrest Pathway in Cells Expressing the Human Papillomavirus Type 16 E7 Oncoprotein

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Received 19 November 1996/Accepted 8 January 1997

Cells expressing human papillomavirus type 16 (HPV-16) E7, similar to those which express HPV-16 E6, are resistant to a p53-mediated G_1 growth arrest. We examined the p53-mediated DNA damage response pathway in E7-expressing cells to determine the mechanism by which E7-containing cells continue to cycle. In response to DNA damage, no dramatic difference was detected in G_1- or S-phase cyclin or cyclin-dependent kinase (Cdk) levels when E7-expressing cells were compared to the parental cell line, RKO. Furthermore, Cdk2 kinase activity was inhibited in both RKO cells and E7-expressing cells, while Cdk2 remained active in E6-expressing cells. However, the steady-state levels of pRB and p107 protein were substantially lower in E7-expressing cells than in the parental RKO cells or E6-expressing cells. There was no reduction in pRB mRNA levels, but the half-life of pRB in E7-expressing cells was markedly shorter. Infection of primary human foreskin keratinocytes with recombinant retroviruses expressing HPV-16 E7 resulted in a decrease in pRB protein levels, indicating this phenomenon is a consequence of E7 expression, not of immortalization or transformation. These data strongly suggest E7 interferes with the stability of pRB and p107 protein. We propose that the removal of these components of the p53-mediated G_1 growth arrest pathway in E7-expressing cells contributes to the ability of E7 to overcome a p53-mediated G_1 growth arrest.

Papillomaviruses are small, double-stranded DNA viruses which induce papillomas, or warts, in many higher vertebrate species. Most papillomaviruses have a specific tropism for squamous epithelial cells. A subset of human papillomaviruses (HPVs) causes a variety of malignancies of the anogenital mucosa. The low-risk viruses, such as HPV type 6 (HPV-6) and HPV-11, have been associated with lesions that have a low probability for malignant progression, such as genital warts (condyloma acuminata). The high-risk types, including HPV-16 and HPV-18, cause squamous intraepithelial lesions that can progress to carcinoma in situ and invasive, cervical carcinoma (reviewed in reference 57).

In most cases where viral infection is associated with the development of cancer, the virus has integrated into the host genome. Integration occurs such that there is disruption of the E2 and/or E1 genes, encoding the major viral regulatory proteins, resulting in deregulated expression of the E6 and E7 viral oncoproteins. Analysis of HPV-positive cervical carcinoma tissues or cell lines derived from them revealed that the E6 and E7 genes are consistently expressed. These data suggest that continued expression of these proteins is important for the initiation and maintenance of the transformed state (reviewed in reference 39). This is consistent with the finding that E6 and E7 are the only two viral genes necessary and sufficient for the efficient immortalization of primary human genital epithelial cells, the normal host cells of these viruses (21, 38).

The mechanisms by which these two viral proteins contribute to uncontrolled cell growth and differentiation are not yet fully understood. The high-risk HPV E6 viral oncoprotein has been shown to interact with and to trigger the degradation of the tumor suppressor protein p53 (44, 53). This is accomplished by the association of high-risk HPV E6 with the cellular protein E6-AP, which in turn targets the p53 protein for degradation by ubiquitin-mediated proteolysis (26). As the absence of p53 function has been correlated with an increased susceptibility to develop cancers (23), the ability of HPV E6 to decrease p53 levels is likely a major factor in its transforming activity. In addition, p53 has been implicated in mediating a G_1 arrest in response to ionizing radiation and DNA damage (30). The absence of this checkpoint control in the presence of HPV E6 may lead to the replication of damaged DNA and the accumulation of chromosomal abnormalities (31, 54), a hallmark of many cancers (19). E6 can also interact with several other cellular proteins (28), including E6BP, a putative calcium binding protein (9).

The oncogenicity of HPV-16 E7 is partially attributed to its ability to bind the retinoblastoma tumor suppressor protein (pRB) and related pRB family members, p107 and p130 (reviewed in reference 37). HPV-16 E7 preferentially binds to the G_0/G_1-specific, hypophosphorylated form of pRB (15). Complexes containing pRB, or other pRB family members, and the cellular transcription factor E2F act as transcriptional repressors of E2F-responsive genes (52). Phosphorylation of pRB near the G_1/S boundary leads to the release of E2F and subsequent transactivation of E2F-responsive genes, which is required for entry into S phase (reviewed in reference 1).

Interaction of high-risk HPV E7 proteins with pRB also inactivates complexes between pRB family members and E2F (8, 40), thus abrogating the delicate, cell cycle-dependent regulation of the transcriptional activity of E2F. This is thought to be an important way in which E7 contributes to deregulation of the cell cycle. There is increasing evidence, however, that E7 is likely to contribute to tumorigenesis in ways beyond this well-characterized interaction with pRB. Although the ability to interact with pRB and the related proteins p107 and p130 is important for the transforming potential of HPV-16 E7, substantial evidence suggests this property is not sufficient for transformation. Certain E7 mutants which are competent for pRB binding are unable to efficiently transform (4, 16, 43). The

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amino-terminal E7 sequences that are similar to the conserved region 1 of adenovirus E1A contribute to cellular transformation independent of pRB binding (4, 7, 16, 43). The carboxyl-terminal Cys-X-X-Cys motifs are also important for the transformation of primary rodent cells (33), immortalization of primary human foreskin keratinocytes (HFKs) (27), and the disruption of the E2F-1–pRB complex (25, 55). This domain of E7 functions as a dimerization domain (10, 33, 58) and can mediate interactions with members of the AP-1 family of transcription factors (3).

The ability of cells expressing HPV-16 E6 or HPV-16 E7 to override a p53-mediated G_1 growth arrest has been well documented (13, 22, 47, 50). One of the initial cellular responses to DNA damage is an increase in the levels of p53. Because p53 is able to transcriptionally activate negative growth regulatory genes, including the gene for the cyclin-dependent kinase (Cdk) inhibitor $p21^{WAF1/Cip1}$ (17), this G_1 arrest involves the inhibition of cyclin-Cdk complexes, including those that normally phosphorylate and inactivate the G_1-specific, growth-suppressive, hypophosphorylated form of pRB (reviewed in reference 20). The ability of E6-expressing cells to overcome a p53-mediated G_1 growth arrest is presumably due to the ability of high-risk HPV E6 proteins to target the p53 protein for degradation (29). However, the mechanism by which E7-expressing cells override the p53-mediated G_1 growth arrest has not yet been clearly defined.

Here we have analyzed components of the cell cycle machinery in E6- or E7-expressing clones of the human colon carcinoma cell line RKO (47) to elucidate the response of E7-expressing cells to a p53-mediated G_1 growth arrest. Previous reports suggested that the HPV-16 E7 oncoprotein is able to activate cell cycle progression downstream of the cyclin-Cdk complexes in a p53-induced G_1 block (13, 22, 47). We report that levels of critical substrates of G_1- and S-phase-specific Cdk complexes, pRB and p107, are decreased as a result of HPV-16 E7 expression. We propose that removal of these negative growth regulators contributes to the ability of E7-expressing cells to override a p53-mediated G_1 growth arrest.

MATERIALS AND METHODS

Cell lines and culture. The human colon carcinoma cell line RKO expresses apparent wild-type pRB and p53 proteins. Two clonal cell populations which express the HPV-16 E7 oncoprotein (RKO 7.6 and RKO 7.14) and two clonal cell populations which express the HPV-16 E6 oncoprotein (RKO 10.1 and RKO 10.2) were kind gifts from Kathy Cho (Johns Hopkins University, Baltimore, Md.) (47). RKO cells were grown in RPMI 1640 (Gibco BRL) supplemented with 10% fetal bovine serum, 50 U of penicillin per ml, and 50 μg of streptomycin per ml. The RKO clones were grown in McCoy's 5A medium (Gibco BRL) supplemented with 5% fetal calf serum, 50 U of penicillin per ml, 50 μg of streptomycin per ml, and 500 μg of Geneticin (Gibco BRL) per ml.

Primary human keratinocytes were prepared from neonatal foreskins as described previously (46) and grown in a serum-free keratinocyte growth medium (Gibco BRL).

The human myeloid leukemia cell line ML-1 was a kind gift from Ed Harlow (MGH Cancer Center, Charlestown, Mass.). The cell line was maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 50 U of penicillin per ml, and 50 μg of streptomycin per ml.

Packaging cell lines (PA317) producing recombinant retroviruses (LXSN, HPV-16 E7, and HPV-16 E7 mutants H2P and C24G) were gifts from Denise Galloway (Fred Hutchinson Cancer Center, Seattle, Wash.) (18). The cell lines were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 50 U of penicillin per ml, and 50 μg of streptomycin per ml, and recombinant retroviruses were prepared by using standard methods (36). HFKs were infected as described previously (18).

Actinomycin D treatment and cell cycle analysis. DNA damage was initiated by treatment with 2.5 nM actinomycin D (Sigma) for 24 h. The cells were treated at approximately 70% confluence. Afterwards, the cells were harvested and lysates were prepared for immunoblot analysis. Cell cycle analysis was performed after the cells were stained with propidium iodide. Briefly, cells were trypsinized and washed several times in phosphate-buffered saline. The cells were then fixed in 100% ethanol for at least 1 h. The cells were washed again in phosphate-buffered saline and treated with 10 μg of RNase H for 1 h at 37°C. The cells were then stained with 0.05 mg of propidium iodide per ml for a minimum of 30 min at 25°C. Fluorescence-activated cell sorting (FACS) analysis was performed and data were analyzed with the CELLFIT program (Becton Dickinson). Approximately 10,000 events were included in each analysis.

Immunological methods. Cell lysates for immunoprecipitation and immunoblot analysis were prepared by incubating the cells in 0.1% Nonidet P-40 (NP-40) lysis buffer (250 mM NaCl, 0.1% NP-40, 50 mM Tris-HCl, 5 mM EDTA, 1 mM dithiothreitol [DTT], 0.5 mM NaF, 0.01% phenylmethylsulfonyl fluoride, 45 nM sodium orthovanadate, 1 μg each of aprotinin and leupeptin per ml [pH 7.0]) at 4°C for 30 min. Cells were then scraped from the plates with a rubber policeman, and extracts were cleared by centrifugation at 15,000 × *g* for 15 min. Protein concentrations were determined by the Bradford method (Bio-Rad). For immunoprecipitations, cell lysates were incubated with the appropriate antibody for 2 to 4 h at 4°C. Immunocomplexes were collected by using protein G-agarose (Gibco BRL) in cases where the primary antibodies were not already conjugated to agarose. Samples were then boiled in sodium dodecyl sulfate (SDS)-containing sample buffer and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto polyvinylidene difluoride membranes (Immobilon P; Millipore). Membranes were blocked in TNET buffer (200 mM Tris HCl, 1 M NaCl, 50 mM EDTA, 0.1% Tween 20 [pH 7.5]) containing 5% nonfat dry milk and probed with the appropriate antibody according to standard protocols. Enhanced chemiluminescence was used to detect antigen-antibody complexes (SuperSignal, Pierce; ECL, Amersham). In cases where evaluation of quantitative differences was necessary, short as well as long exposures were analyzed to ensure that the differences observed were within the linear range of the X-ray film.

Antibodies. Monoclonal antibodies against $p21^{WAF1/Cip1}$ (CP36 and CP68) were gifts from B. Dynlacht (Harvard University, Cambridge, Mass.). Monoclonal antibodies against p107 (SD9, SD4, SD6, and SD2) and pRB (XZ77) were gifts from N. Dyson (MGH Cancer Center). The monoclonal pRB antibody G3-245 was purchased from PharMingen. Monoclonal antibodies against p130 were gifts from J. DeCaprio (Dana-Farber Cancer Institute, Boston, Mass.). The cyclin E antibody (HE12), cyclin A antibody (BF683), cyclin D1 antibody (HD11), Cdk2 antibody (M2), Cdk4 antibody (C-22), and Cdk6 antibody (C-21) were purchased from Santa Cruz Biotechnologies. The p53 antibody (DO-1; Ab6) was purchased from Oncogene Science. Monoclonal antibodies against E7 were produced by J. DeCaprio using standard methods and glutathione *S*-transferase (GST)–E7 fusion protein as an antigen. Briefly, antibodies were generated by immunizing RBF/Dnj mice with GST-E7 protein and fusing splenocytes of positive animals to NS1 cells. Secondary antibodies used in immunoblot analysis, horseradish peroxidase-linked sheep anti-mouse and donkey anti-rabbit antibodies, were purchased from Amersham.

Kinase assays. Histone H1 kinase assays were performed according to standard protocols, using established immunological reagents. Briefly, immunoprecipitations were carried out at 4°C for 2 to 4 h, using the appropriate antibody. Immunocomplexes were washed three times in 0.1% NP-40 lysis buffer and twice in kinase buffer (25 mM Tris HCl, 10 mM $MgCl_2$, 1 mM DTT, 100 μM ATP [pH 7.5]). Kinase reactions were initiated by the addition of 1 μg of histone H1 (Boehringer Mannheim) and 10 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol; NEN), and reaction mixtures were incubated at 37°C for 30 min. Products were analyzed by SDS-PAGE and autoradiography. To avoid nonspecific background, extracts were precleared with normal rabbit serum and protein A-Sepharose.

Cdk4 and Cdk6 kinase assays were performed as described previously (35). For these assays, cells were lysed in DIP buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% [vol/vol] glycerol, 0.1% Tween 20, 1 mM DTT, 0.5 mM NaF, 0.01% phenylmethylsulfonyl fluoride, 45 nM sodium orthovanadate, 1 μg each of aprotinin and leupeptin per ml [pH 7.2]). Extracts were prepared and protein concentrations were determined as described above. After immunoprecipitation with either Cdk4- or Cdk6-specific antibodies, immunocomplexes were collected by using protein A-Sepharose (Pharmacia). Complexes were then washed three times in kinase buffer (50 mM HEPES, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT, 100 μM ATP [pH 7.4]). Reactions were initiated by the addition of 10 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol; NEN) and a carboxyl-terminal fragment of pRB fused to GST (obtained from E. Harlow) as a substrate (35). Kinase assays were performed at 37°C for 30 min, and products were analyzed by SDS-PAGE and autoradiography.

Northern blot analysis. Total RNA was isolated from subconfluent RKO cells and clonal lines according to standard procedures (11). RNA (30 μg) was size fractionated on a 1% agarose–morpholino propanesulfonic acid (MOPS)-formaldehyde gel and transferred to a nylon membrane (Hybond N, Amersham) according to the manufacturer's protocol. Hybridization probes were generated by random priming.

Protein half-life determination. RKO, RKO 7.6, and RKO 10.1 cells were plated on 60-mm-diameter dishes (1.4×10^6 cells/dish). The cells were treated with 20 μg of cycloheximide (Actidione; Fluka) per ml for the times indicated. After treatment, the cells were harvested and lysed in 0.1% NP-40 lysis buffer. Samples (100 μg) were analyzed by SDS-PAGE and immunoblotting as described above.

Peptide inhibitor treatments. RKO, RKO 7.6, and RKO 10.1 cells were plated onto 100-mm-diameter dishes and treated for the times indicated with various inhibitors. MG132 treatment was performed for 6 h at a concentration of 10 μM (6, 41). MG132 was purchased from Peptides International (Louisville, Ky.).

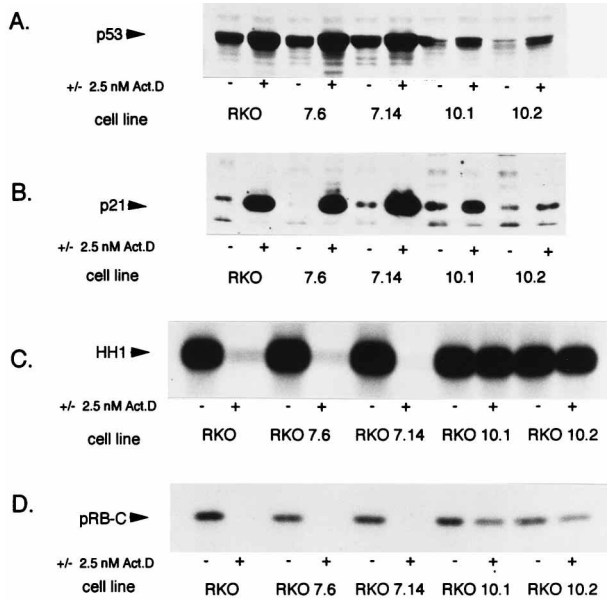

FIG. 1. Induction of p53 and $p21^{WAF1/Cip1}$ and activity of Cdk2 in RKO cells, RKO cells expressing E7 (RKO 7.6 and RKO 7.14), and RKO cells expressing E6 (RKO 10.1 and RKO 10.2) in response to treatment with actinomycin D. Cells were treated (+) or not treated (−) with 2.5 nM actinomycin D (Act. D) for 24 h at approximately 70% confluence. Cells were lysed in 0.1% NP-40 lysis buffer, and samples (100 μg) were analyzed by SDS-PAGE, immunoblotting, and enhanced chemiluminescence. Blots were probed with the p53 monoclonal antibody Ab6 (A) or with the $p21^{WAF1/Cip1}$ monoclonal antibody CP36 (B). Cdk2-associated kinase activity was determined as described in Materials and Methods, using histone H1 (C) or a C-terminal fragment of pRB (D) as the substrate.

YVAD-CHO treatment was performed for 12 h at a concentration of 50 μM. YVAD-CHO and YVAD-CMK were purchased from Bachem Bioscience. Treated cells were harvested and lysed in 0.1% NP-40 lysis buffer, and samples (100 μg) were analyzed by SDS-PAGE and immunoblotting as described above.

RESULTS

Accumulation of p53 and $p21^{WAF1/Cip1}$ in actinomycin D-treated RKO cells, E7-expressing cells, and E6-expressing cells. To confirm that the E7-expressing RKO cells continue to cycle after being subjected to DNA-damaging agents, RKO cells, RKO cells expressing E7 (clones 7.6 and 7.14), and RKO cells expressing E6 (clones 10.1 and 10.2) were treated with 2.5 nM actinomycin D and harvested, and their cell cycle distribution was analyzed by FACS analysis. The relative decrease in the S-phase fraction (percent S-phase cells untreated/percent S-phase cells treated) in the E7-expressing RKO clones (RKO 7.6 and RKO 7.14) was 4.4, substantially less than in parental cells (9.9). The values for the E6-expressing cell lines RKO 10.1 and 10.2 were 2.6 and 1.4, respectively. These numbers are averages of four independent experiments and are essentially identical to a previously published analysis of the same set of cells, where gamma irradiation was used to inflict DNA damage (47).

The accumulation of p53 and $p21^{WAF1/Cip1}$ protein in these cells in response to DNA damage was also determined by immunoblot analysis. Significant increases of both p53 and $p21^{WAF1/Cip1}$ proteins were seen in RKO, RKO 7.6, and RKO 7.14 cells. E6-expressing cells contained decreased initial levels of p53 and $p21^{WAF1/Cip1}$; therefore, although actinomycin D treatment resulted in increases in the levels of these two proteins, the levels remained significantly lower than in parental RKO cells and E7-expressing cells (Fig. 1A and B). These data demonstrate that the E7-expressing cells have maintained the ability to accumulate p53 in response to DNA damage and that the p53 protein is in turn able to transcriptionally activate expression of one of its known targets, $p21^{WAF1/Cip1}$.

Enzymatic activity of cyclin-Cdk complexes in actinomycin D-treated cells. Because both E6- and E7-expressing RKO cells continue to cycle after exposure to DNA-damaging agents, we hypothesized that the enzymatic activity of various G_1- and S-phase Cdks may be less inhibited in these cell populations after DNA damage. Cdk2-, cyclin A-, and cyclin E-associated kinase activities, however, were substantially inhibited in both E7-expressing RKO clones, 7.6 and 7.14, upon treatment with actinomycin D. In fact, the degree of inhibition was indistinguishable from that observed in the parental RKO cells which have growth arrested (Fig. 1C and D). Similar results were obtained with both histone H1 and a C-terminal fragment of pRB as substrates. Consistent with decreased $p21^{WAF1/Cip1}$ levels, the activity of these kinases in E6-expressing cells was unaffected (Fig. 1C and D). Upon actinomycin D treatment, association of $p21^{WAF1/Cip1}$ with Cdk2 was seen in RKO cells and both E7-expressing clones but was not detected in E6-expressing clones, verifying that inhibition of kinase activity was at least in part due to the association of $p21^{WAF1/Cip1}$ with these kinases (data not shown). Cdk4 and Cdk6 kinase activities were also determined in response to actinomycin D treatment in these cells. Activities of these kinases were unaffected by actinomycin D treatment in all cell lines tested, although $p21^{WAF1/Cip1}$ was found to be associated with Cdk6 after DNA damage in the parental RKO cells and in both E7-expressing clones (data not shown).

Steady-state levels of cell cycle regulatory proteins in response to actinomycin D treatment. Because E7-expressing cells continue to progress through the cell cycle in the presence of high levels of p53 and $p21^{WAF1/Cip1}$ and inhibited Cdk2 kinase activity, we determined whether the levels of cell cycle regulatory proteins were altered in E7-expressing cells in response to DNA damage. We analyzed the levels of cyclins A, E, and D1 and of Cdk2, Cdk4, and Cdk6 by immunoblotting (Fig. 2). The initial steady-state levels of these proteins were similar in RKO, RKO 7.6, and RKO 7.14 cells. Although the steady-state levels of several of these proteins did change upon treatment with actinomycin D, no specific difference was observed in the E7-expressing clones compared to the parental RKO cell line. In brief, levels of cyclin E were induced, cyclin A and Cdk6 levels were slightly decreased, and cyclin D1, Cdk2, and Cdk4 levels stayed the same in response to actinomycin D treatment (Fig. 2).

Next we determined the steady-state levels of known substrates of G_1/S-phase cyclin-Cdk complexes, namely, pRB and p107. Strikingly, the initial levels of pRB and p107 in E7-expressing cells were significantly lower than in the wild-type RKO cells and the E6-expressing clones (Fig. 3A and B). The decrease in pRB seen in E7-expressing RKO cells is comparable to the decrease in p53 levels in E6-expressing cells (compare Fig. 1A and 3A). Similar results were also observed for the pRB family member p130 (data not shown). Due to the low steady-state level of this protein in these cells, the results are less clear and the experiments will need to be repeated in cells that express higher levels of the protein. Actinomycin D treatment led to a decrease in pRB and p107 levels in parental RKO cells as well as in the E7-expressing clones, as has been described previously (13). The basal levels of pRB in E6-expressing cells were high and decreased to a lesser extent in response to actinomycin D treatment. The accumulation of hypophosphorylated pRB was observed only in the parental RKO cells in response to actinomycin D treatment. Despite

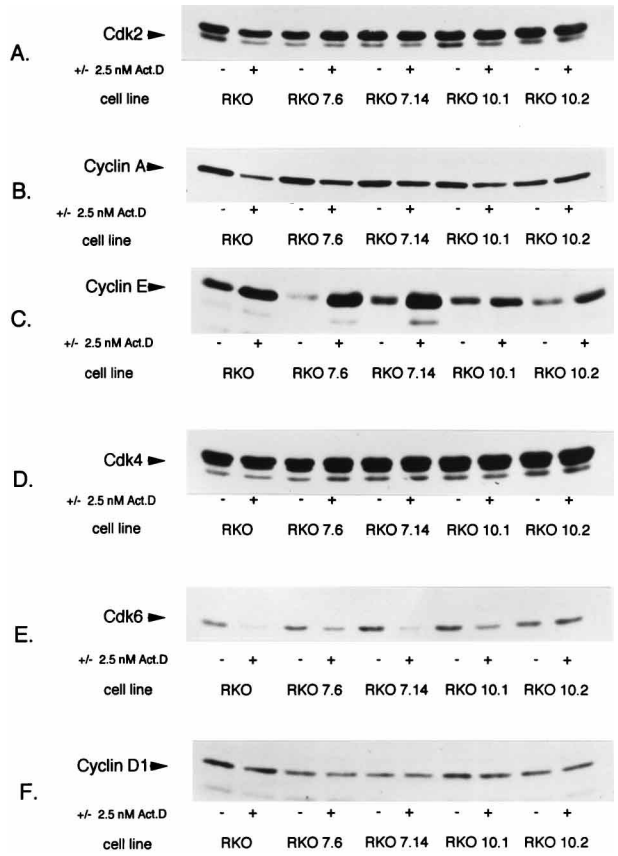

FIG. 2. Steady-state levels of cell cycle regulatory proteins in response to actinomycin D treatment. RKO cells, E7-expressing RKO cells (RKO 7.6 and RKO 7.14), and E6-expressing RKO cells (RKO 10.1 and RKO 10.2) were treated (+) or not treated (−) with actinomycin D (Act. D), and samples were analyzed by immunoblot analysis as described in Materials and Methods. Blots were probed with the Cdk2 antibody M2 (A), cyclin A antibody BF683 (B), cyclin E antibody HE12 (C), Cdk4 antibody C-22 (D), Cdk6 antibody C-21 (E), and cyclin D1 antibody HD11 (F).

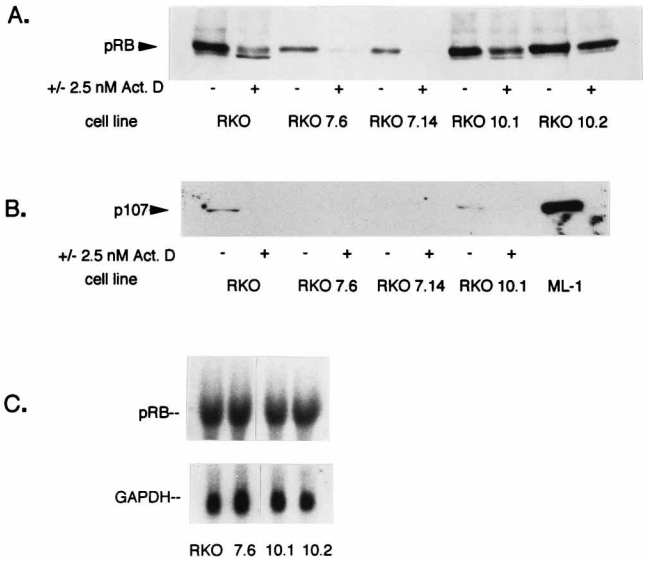

FIG. 3. Analysis of pRB and p107 levels. (A) Immunoblot analysis of pRB from actinomycin D-treated (+) and nontreated (−) RKO cells, RKO cells expressing E7 (RKO 7.6 and RKO 7.14), and RKO cells expressing E6 (RKO 10.1 and RKO 10.2). Actinomycin D (Act. D) treatment was performed and samples were prepared as described in Materials and Methods. The pRB monoclonal antibody G3-245 was used. (B) Immunoblot analysis of p107. The assay was as described above except that a mix of p107 monoclonal antibodies (SD2, SD4, SD6, and SD9) was used. (C) pRB Northern blot analysis. Total RNA was isolated from subconfluent RKO cells, RKO cells expressing E7 (7.6), or RKO cells expressing E6 (10.1 and 10.2). RNA (30 μg) was size fractionated on a 1% agarose–MOPS-formaldehyde gel, transferred to a nylon membrane, and hybridized to a labeled fragment of the pRB gene. Hybridization using a fragment of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was included as a loading control.

similarly low kinase activities, hyperphosphorylated pRB was detected in the E7-expressing clones, consistent with the FACS analysis data, verifying that the cells were continuing to cycle.

Decreased pRB levels are a result of decreased protein stability. Northern blot analysis was performed to determine if the decrease in pRB protein levels was reflected in a concomitant decrease in pRB mRNA levels in E7-expressing cells. No difference in mRNA levels was seen in the E7-expressing clone (RKO 7.6) compared to E6-expressing cells or the parental cell line, RKO (Fig. 3C). This finding demonstrates that the levels of pRB, and presumably p107, protein are altered by a post-transcriptional mechanism. Next we determined whether E7 expression has an effect on the stability of pRB protein. pRB was seen to have a long half-life in RKO and E6-expressing RKO (RKO 10.1) cells (Fig. 4). Since no marked decreases in pRB levels were detected over the 12-h time period assayed, it is estimated that pRB has a half-life of more than 12 h in these cells. This is in agreement with the pRB half-life in mammary epithelial cells determined by pulse-chase analysis (6). In contrast, the half-life of pRB was clearly decreased to less than 12 h in E7-expressing RKO (RKO 7.6) cells (Fig. 4). This result is also consistent with the results of Boyer et al., who report a similar decrease in pRB half-life by pulse-chase analysis of mammary epithelial cells immortalized by E7 (6). As a control, the half-life of p53 was also determined. The steady-state level of p53 was significantly lower in the E6-expressing cells than in the parental RKOs and E7-expressing RKO cells (Fig. 1A and 4). In both RKO cells and the E6-expressing RKO cells, the half-life of p53 was less than 3 h. In contrast, the half-life of p53 in E7-expressing cells was actually increased (Fig. 4).

pRB levels in response to treatments with inhibitors of protein degradation. There is a precedent for HPV oncoproteins directly affecting the stability of an associated cellular protein, specifically, the targeting of p53 for ubiquitin-mediated proteolysis by high-risk HPV E6 proteins (45). Moreover, pRB can be efficiently targeted for ubiquitin-mediated proteolysis in vitro by an E6-E7 chimeric protein containing the pRB binding domain of E7 (44). A recent publication has provided evidence for the involvement of the ubiquitin-dependent protease system in the accelerated degradation of pRB by E7 in immortalized human breast epithelial cells (6). Therefore, we treated RKO cells, E7-expressing RKO cells, and E6-expressing RKO cells with MG132, an inhibitor of the 26S proteosome (41). Although dramatic increases in the levels of p53 were seen in all cell lines tested, only minimal increases of pRB levels were observed in both RKO cells and in RKO cells expressing E7 (Fig. 5A).

It has also been reported that pRB is a substrate for an interleukin-1β-converting enzyme (ICE)-like cysteine protease in response to apoptotic signals (2). Because HPV-16 E7 has been shown to induce apoptosis in transgenic animals (24, 42) and apoptotic-like responses in tissue culture cells (54), we exposed RKO cells, E7-expressing RKO cells, and E6-expressing RKO cells to the inhibitors of the ICE family of cysteine

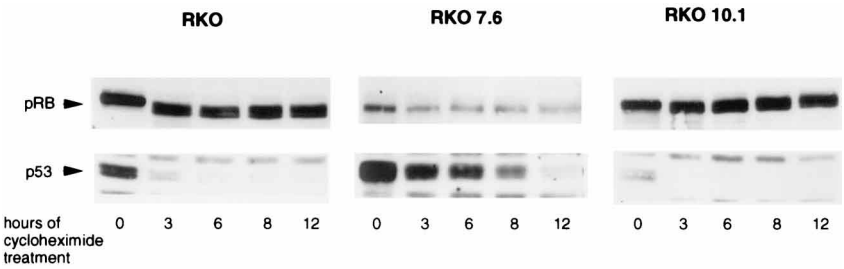

FIG. 4. Determination of pRB half-life. RKO cells, E7-expressing RKO cells (RKO 7.6), and E6-expressing RKO cells (RKO 10.1) were treated with cycloheximide (20 μg/ml) for the times indicated. Cells were harvested and lysed in 0.1% NP-40 lysis buffer, and samples (100 μg) were analyzed by SDS-PAGE and immunoblotting. pRB and p53 immunoblots for RKO 7.6 and RKO 10.1 samples were taken from the same film for accurate comparison. The pRB blot was probed with monoclonal pRB antibody XZ77, and the p53 blot was probed with monoclonal p53 antibody Ab6.

proteases, YVAD-CHO and YVAD-CMK. We detected a reproducible two- to threefold increase in pRB levels in response to treatment with YVAD-CHO. This effect was observed in both parental cells and E7-expressing RKO cells (Fig. 5B). This finding suggests that apoptotic cleavage may contribute to the decreased levels of pRB in E7-expressing RKO cells but that this pathway may play a role in the regulated turnover of pRB in the parental RKO cells as well. These data indicate that multiple pathways are involved in the regulated turnover of pRB in the cell, and it is not clear which one, if any, is targeted by E7 in these cells.

Levels of pRB protein in primary HFKs expressing HPV-16 E7. To determine if decreases in pRB levels are coincident with E7 expression in cells, we infected primary HFKs with recombinant retroviruses expressing HPV-16 E7. Infected cells were selected with G418 and harvested at different times postinfection. E7 expression was verified by immunoblot analysis and was first detected at day 8 postinfection. Increasing steady-state levels were detected thereafter, and pRB levels decreased in parallel with increasing E7 expression (data not shown). At day 14 postinfection, the steady-state levels of pRB were found to be dramatically decreased specifically in cells expressing E7, suggesting that the decreased pRB levels are a result of E7 expression (Fig. 6).

In parallel, we also infected primary HFKs with retroviruses expressing two previously characterized mutants of E7. One of the mutants, C24G, has the cysteine residue in the core pRB binding site mutated to a glycine residue and has a severely impaired capacity to interact with pRB (5, 16). A second mutant, H2P, has a mutation in the amino-terminal domain, which is homologous to conserved region 1 of adenovirus E1A, and can efficiently interact with pRB (4). Both of these mutants are impaired for cellular transformation (4, 16) and negative for abrogation of p53-mediated G_1 growth arrest signals and transforming growth factor β-mediated growth arrest signals (12). Both mutants of E7 were unable to affect pRB stability to the same degree as wild-type HPV-16 E7 (Fig. 6). Not only do these data correlate the ability to abrogate a p53-mediated G_1 growth arrest with the ability of E7 to affect pRB stability, but more surprisingly, they also suggest that sequences of E7 outside of the core pRB binding domain contribute to this property of E7.

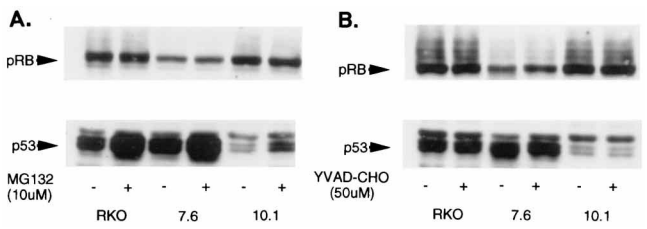

FIG. 5. pRB levels in response to inhibitors of protein degradation. (A) RKO cells, E7-expressing RKO cells (7.6), and E6-expressing cells (10.1) were treated with the proteosome inhibitor MG132. Cells were treated with 10 μM MG132 for 6 h at approximately 70% confluence. Cells were lysed in 0.1% NP-40 lysis buffer, and samples (100 μg) were analyzed by SDS-PAGE and immunoblotting. pRB and p53 immunoblots are shown for each cell line. (B) RKO cells, E7-expressing RKO cells (7.6), and E6-expressing RKO cells (10.1) were treated with a specific peptide inhibitor (YVAD-CHO) of the ICE family of cysteine proteases. Cells were treated with 50 μM YVAD-CHO for 12 h at approximately 70% confluence. Samples were prepared and analyzed as described above. The pRB blot was probed with monoclonal pRB antibody G3-245, and the p53 blot was probed with monoclonal p53 antibody Ab6.

DISCUSSION

Several reports have shown that E7-expressing cells are partially resistant to a p53-mediated G_1 growth arrest. This property of E7 has been observed in various cell types and in response to different inducers of DNA damage (13, 22, 47, 50). Indirectly, these data indicated a role for pRB downstream of p53 in the DNA damage response pathway (reviewed in reference 20). It was proposed that due to E7's ability to bind pRB and related proteins p107 and p130, E2F was released and resumption of the cell cycle ensued. This model predicts that upon inhibition of Cdks, hypophosphorylated pRB will accumulate in E7-expressing clones and that the cells will continue to cycle due to association of E7 with hypophosphorylated pRB. However, hyperphosphorylated pRB was detected in DNA-damaged, E7-expressing cells (Fig. 3A). This form of pRB is not efficiently bound by E7. Therefore, the model that E7 may allow cell cycle progression simply by binding to the growth-suppressive form of pRB alone cannot sufficiently explain the ability of these cells to continue to cycle in response to DNA damage.

In an effort to determine the mechanism by which E7-expressing cells continue to cycle in response to a p53-mediated G_1 arrest, we analyzed various components of the cell cycle machinery in E7-expressing cells and compared them to those in cells which do not express E7. In response to actinomycin D treatment, the p53 and $p21^{WAF1/Cip1}$ levels increased in E7-expressing cells as was seen in the parental cell line, RKO (Fig. 1A and B). In addition, we found that the protein levels of G_1- and S-phase cyclins and Cdks were the same in the E7-expressing cells as in the parental RKO cells (Fig. 2). Due to the fact that E7-expressing cells continue to cycle in response to DNA damage, we predicted that there may be less inhibition of Cdk activity in these cells. However, we found that Cdk2 kinase activity was inhibited to an extent similar to that of the growth-arrested parental cells (Fig. 1C and D). Moreover,

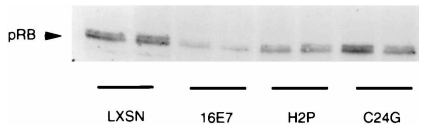

FIG. 6. pRB levels in cells infected with recombinant retroviruses expressing HPV-16 E7. Primary HFK were infected with the control retrovirus LXSN and recombinant retroviruses expressing HPV-16 E7 (16E7) or mutants of E7 (H2P, and C24G). Infected cells were selected for resistance to G418 and harvested 14 days postinfection. Cells were lysed in 0.1% NP-40 lysis buffer, and samples (100 μg) were analyzed by SDS-PAGE and immunoblot analysis for pRB. Grouped lanes represent duplicate infections. The pRB blot was probed with monoclonal pRB antibody G3-245.

$p21^{WAF1/Cip1}$ was complexed with Cdk2 in both E7-expressing cells and parental RKO cells after treatment with actinomycin D (data not shown). Cdk4 and Cdk6 activities were not substantially inhibited in any of the cell lines tested (data not shown).

E7 has been reported to associate with cyclin A, cyclin E, and a cellular protein kinase activity (15, 34, 49). This kinase has been identified as Cdk2 (49). The nature of this association has not been conclusively defined, although McIntyre et al. recently reported the requirement for p107 in the detection of an association between HPV-18 E7 and cyclin E (34). E7-containing Cdk complexes may be differentially susceptible to inhibition by $p21^{WAF1/Cip1}$ or other kinase inhibitors induced by DNA damage. It has been demonstrated that another viral oncoprotein, adenovirus E1A, can alter the susceptibility of Cdk2 containing complexes to inhibition by the Cdk inhibitor $p27^{Kip1}$ in response to transforming growth factor β treatment of Mv1Lu cells (32). In our analyses of cyclin A-, cyclin E-, and Cdk2-containing kinase complexes in E7-expressing cells, however, we were unable to detect the presence of E7 in any of these kinase complexes (data not shown). Thus, our results are similar to those reported by McIntyre et al., who, using the same cyclin E antibody (HE12), also failed to detect E7 by coimmunoprecipitation. In that study, the cyclin E-E7 complex was detected only when an E7-specific antibody was used. This finding suggests that in the current study, either the cyclin or Cdk antibodies used for these analyses are not able to efficiently precipitate E7-containing complexes or E7 is unable to associate with these kinase complexes due to the low levels of p107 protein.

We attempted to directly determine E7-associated kinase activity in E7-expressing RKO cells by using several E7-specific, monoclonal antibodies. Although each antibody precipitated considerable amounts of histone H1 kinase activity from both actinomycin D-treated and nontreated cells, each of these antibodies also precipitated histone H1 kinase activity from non-E7-containing RKO cells. Additional experiments are currently being performed to characterize in detail the molecular components, enzymatic activities, and susceptibility to Cdk inhibitors of this E7-associated kinase activity.

While our analysis did not reveal major differences in steady-state levels or enzymatic activities of cyclin-Cdk complexes between the growth-arrested RKO and the cycling E7-expressing RKO clones, we detected significant differences in the steady-state levels of known substrates of these cyclin-Cdk complexes, namely, pRB and the related proteins p107 and p130. We have shown that expression of HPV-16 E7 in RKO colon carcinoma cells results in decreased levels of pRB, p107, and apparently p130 as well (Fig. 3A and B and data not shown). The decrease in pRB protein levels is not reflected in a decrease in mRNA levels but is due to a decreased stability of the pRB protein, specifically in E7-expressing cells (Fig. 3C and 4). In addition, infection of HFKs with a recombinant retrovirus expressing HPV-16 E7 leads to a decrease in pRB protein levels, substantiating the hypothesis that the decreased pRB and p107 levels are a consequence of E7 expression (Fig. 6). Decreases in the steady-state levels of pRB in the presence of HPV-16 E7 have also been noted in E7-immortalized mammary epithelial cells (51), in E7-expressing uroepithelial cells (56), and in E7-expressing human fibroblasts (54) and keratinocytes (13). However, this is the first instance in which decreased levels of p107 have been reported as a consequence of E7 expression.

Interfering with the stability of proteins may prove to be an emerging strategy by which viral oncoproteins can functionally abrogate target proteins. It has been shown that simian virus 40 large T antigen directly affects the phosphorylation state of p130 and p107 (48). In addition, these authors comment that there may be a decrease in p130 protein in T-antigen-expressing cells and suggest that T antigen might affect the stability of hyperphosphorylated p130.

A recent study by Boyer et al. showed that the half-life of pRB was decreased in E7-immortalized mammary epithelial cells and provided evidence for the involvement of the ubiquitin-mediated proteolysis pathway in pRB degradation in these cells (6). We performed similar experiments, and blocking of the ubiquitin proteolysis pathway by using an inhibitor of the 26S proteosome, MG132, had no marked effect on pRB levels in parental or E7-expressing RKO cells. In contrast, significant increases in p53 levels were observed in all cell lines tested, indicating that this pathway is indeed active in these cells and inhibitable by the appropriate compounds. A modest increase in pRB was observed in both parental and E7-expressing RKO cells after treatment with a peptide inhibitor specific for the ICE family of cysteine proteases. The effect of this inhibitor was not tested in the system of Boyer et al. (6). Furthermore, in agreement with Demers et al. (14), we observed a dramatic stabilization of p53 in E7-expressing cells (Fig. 4), which was not detected in mammary epithelial cells, indicating another difference between the two systems (6). Additional studies are required to biochemically define the nature of the proteolytic system(s) utilized in the degradation of pRB and the mechanism by which it is targeted by HPV-16 E7.

Infection with recombinant retroviruses expressing HPV-16 E7 showed that pRB degradation is a consequence of E7 expression, not of immortalization or transformation. Interestingly, our data suggest regions of E7 outside the core pRB binding site are necessary for this effect, indicating the exciting possibility that E7 mutations which are not directly involved in pRB binding in vitro may have a critical defect in targeting pRB in the cell.

The ability of HPV-16 E7 to interfere with accumulation of pRB may be an important viral strategy to make the targeting of pRB by E7 more efficient. One of the main functions of E7 in an HPV infection is to allow viral replication by ensuring continued cellular DNA synthesis in normally growth arrested keratinocytes. The amounts of E7 that are expressed during infection of cells with high-risk HPVs are generally quite low and may not suffice to stoichiometrically bind to and functionally abrogate the entire intracellular pool of hypophosphorylated pRB. In future experiments, we will attempt to elucidate the mechanism behind the ability of E7 to decrease the levels of pRB and p107 in the cell.

ACKNOWLEDGMENTS

We thank Kathy Cho for the RKO cell clones expressing E7 and E6, Denise Galloway for the E7-expressing retroviral vectors, Nick Dyson,

James DeCaprio, and Brian Dynlacht for providing monoclonal antibodies, and Rhoda M. Alani for the preparation of human keratinocytes. We also thank Nick Dyson, Yang Shi, Kathy Cho, and members of the Münger lab for suggestions and critical reading of the manuscript.

This work was supported by grant CA 66980 from the National Cancer Institute. K.M. is the recipient of Junior Faculty Research Award JFRA-597 from the American Cancer Society.

REFERENCES

1. **Adams, P. D., and W. G. Kaelin, Jr.** 1995. Transcriptional control by E2F. Semin. Cancer Biol. **6:**99–108.
2. **An, B., and Q. P. Dou.** 1996. Cleavage of retinoblastoma protein during apoptosis: an interleukin 1 beta-converting enzyme-like protease as candidate. Cancer Res. **56:**438–442.
3. **Antinore, M. J., M. J. Birrer, D. Patel, L. Nader, and D. J. McCance.** 1996. The human papillomavirus type 16 E7 gene product interacts with and transactivates the AP1 family of transcription factors. EMBO J. **15:**1950–1960.
4. **Banks, L., C. Edmonds, and K. Vousden.** 1990. Ability of the HPV16 E7 protein to bind RB and induce DNA synthesis is not sufficient for efficient transforming activity in NIH 3T3 cells. Oncogene **5:**1383–1389.
5. **Barbosa, M. S., C. Edmonds, C. Fisher, J. T. Schiller, D. R. Lowy, and K. H. Vousden.** 1990. The region of the HPV E7 oncoprotein homologous to adenovirus E1a and SV40 large T antigen contains separate domains for Rb binding and casein kinase II. EMBO J. **9:**153–160.
6. **Boyer, S. N., D. E. Wazer, and V. Band.** 1996. E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteosome pathway. Cancer Res. **56:**4620–4624.
7. **Brokaw, J. L., C. L. Yee, and K. Münger.** 1994. A mutational analysis of the amino terminal domain of the human papillomavirus type 16 E7 oncoprotein. Virology **205:**603–607.
8. **Chellappan, S., V. B. Kraus, B. Kroger, K. Münger, P. M. Howley, W. C. Phelps, and J. R. Nevins.** 1992. Adenovirus E1A, simian virus 40 tumor antigen, and human papillomavirus E7 protein share the capacity to disrupt the interaction between the transcription factor E2F and the retinoblastoma gene product. Proc. Natl. Acad. Sci. USA **89:**4549–4553.
9. **Chen, J. J., C. E. Reid, V. Band, and E. J. Androphy.** 1995. Interaction of papillomavirus E6 oncoproteins with a putative calcium-binding protein. Science **269:**529–531.
10. **Clemens, K. E., R. Brent, J. Gyuris, and K. Munger.** 1995. Dimerization of the human papillomavirus E7 oncoprotein in vivo. Virology **214:**289–293.
11. **Davis, L. G., M. D. Dibner, and J. F. Battey.** 1986. Basic methods in molecular biology. Elsevier Science, New York, N.Y.
12. **Demers, G. W., E. Espling, J. B. Harry, B. G. Etscheid, and D. A. Galloway.** 1996. Abrogation of growth arrest signals by human papillomavirus type 16 E7 is mediated by sequences required for transformation. J. Virol. **70:**6862–6869.
13. **Demers, G. W., S. A. Foster, C. L. Halbert, and D. A. Galloway.** 1994. Growth arrest by induction of p53 in DNA damaged keratinocytes is bypassed by human papillomavirus 16 E7. Proc. Natl. Acad. Sci. USA **91:**4382–4386.
14. **Demers, G. W., C. L. Halbert, and D. A. Galloway.** 1994. Elevated wild-type p53 protein levels in human epithelial cell lines immortalized by the human papillomavirus type 16 E7 gene. Virology **198:**169–174.
15. **Dyson, N., P. Guida, K. Münger, and E. Harlow.** 1992. Homologous sequences in adenovirus E1A and human papillomavirus E7 proteins mediate interaction with the same set of cellular proteins. J. Virol. **66:**6893–6902.
16. **Edmonds, C., and K. H. Vousden.** 1989. A point mutational analysis of human papillomavirus type 16 E7 protein. J. Virol. **63:**2650–2656.
17. **ElDeiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler, and B. Vogelstein.** 1993. WAF1, a potential mediator of p53 tumor suppression. Cell **75:**817–825.
18. **Halbert, C. L., W. Demers, and D. A. Galloway.** 1991. The E7 gene of human papillomavirus type 16 is sufficient for immortalization of human epithelial cells. J. Virol. **65:**473–478.
19. **Hartwell, L.** 1992. Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. Cell **71:**543–546.
20. **Hartwell, L. H., and M. B. Kastan.** 1994. Cell cycle control and cancer. Science **266:**1821–1828.
21. **Hawley-Nelson, P., K. H. Vousden, N. L. Hubbert, D. R. Lowy, and J. T. Schiller.** 1989. HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. EMBO J. **8:**3905–3910.
22. **Hickman, E. S., S. M. Picksley, and K. H. Vousden.** 1994. Cells expressing HPV16 E7 continue cell cycle progression following DNA damage induced p53 activation. Oncogene **9:**2177–2181.
23. **Hollstein, M., D. Sidransky, B. Vogelstein, and C. C. Harris.** 1991. p53 mutations in human cancers. Science **253:**49–53.
24. **Howes, K. A., L. N. Ransom, D. S. Papermaster, J. G. H. Lasudry, D. M. Albert, and J. J. Windle.** 1994. Apoptosis or retinoblastoma: alternative fates of photoreceptors expressing the HPV-16 E7 gene in the presence or absence of p53. Genes Dev. **8:**1300–1310.
25. **Huang, P. S., D. R. Patrick, G. Edwards, P. J. Goodhart, H. E. Huber, L. Miles, V. M. Garsky, A. Oliff, and D. C. Heimbrook.** 1993. Protein domains governing interactions between E2F, the retinoblastoma gene product, and human papillomavirus type 16 E7 protein. Mol. Cell. Biol. **13:**953–960.
26. **Huibregtse, J. M., M. Scheffner, and P. M. Howley.** 1991. A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18. EMBO J. **10:**4129–4135.
27. **Jewers, R. J., P. Hildebrandt, J. W. Ludlow, B. Kell, and D. J. McCance.** 1992. Regions of human papillomavirus type 16 E7 oncoprotein required for immortalization of human keratinocytes. J. Virol. **66:**1329–1335.
28. **Keen, N., R. Elston, and L. Crawford.** 1994. Interaction of the E6 protein of human papillomavirus with cellular proteins. Oncogene **9:**1493–1499.
29. **Kessis, T. D., R. J. Slebos, W. G. Nelson, M. B. Kastan, B. S. Plunkett, S. M. Han, A. T. Lorincz, L. Hedrick, and K. R. Cho.** 1993. Human papillomavirus 16 E6 expression disrupts the p53-mediated cellular response to DNA damage. Proc. Natl. Acad. Sci. USA **90:**3988–3992.
30. **Lane, D. P.** 1992. Cancer. p53, guardian of the genome. Nature **358:**15–16.
31. **Livingstone, L. R., A. White, J. Sprouse, E. Livanos, T. Jacks, and T. D. Tlsty.** 1992. Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. Cell **70:**923–935.
32. **Mal, A., R. Y. C. Poon, P. H. Howe, H. Toyoshima, T. Hunter, and M. L. Harter.** 1996. Inactivation of $p27^{Kip1}$ by the viral E1A oncoprotein in TGFβ-treated cells. Nature **380:**262–265.
33. **McIntyre, M. C., M. G. Frattini, S. R. Grossman, and L. A. Laimins.** 1993. Human papillomavirus type 18 E7 protein requires intact Cys-X-X-Cys motifs for zinc binding, dimerization, and transformation but not for Rb binding. J. Virol. **67:**3142–3150.
34. **McIntyre, M. C., M. N. Ruesch, and L. A. Laimins.** 1996. Human papillomavirus E7 oncoproteins bind a single form of cyclin E in a complex with cdk2 and p107. Virology **215:**73–82.
35. **Meyerson, M., and E. Harlow.** 1994. Identification of G_1 kinase activity for cdk6, a novel cyclin D partner. Mol. Cell. Biol. **14:**2077–2086.
36. **Miller, A. D., and G. J. Rosman.** 1989. Improved retroviral vectors for gene transfer and expression. BioTechniques. **7(9):**980–988.
37. **Münger, K., and W. C. Phelps.** 1993. The human papillomavirus-E7 protein as a transforming and transactivating factor. Biochim. Biophys. Acta **1155:**111–123.
38. **Münger, K., W. C. Phelps, V. Bubb, P. M. Howley, and R. Schlegel.** 1989. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J. Virol. **63:**4417–4421.
39. **Münger, K., M. Scheffner, J. M. Huibregtse, and P. M. Howley.** 1992. Interactions of HPV E6 and E7 with tumour suppressor gene products. Cancer Surv. **12:**197–217.
40. **Pagano, M., M. Dürst, S. Joswig, G. Draetta, and P. Jansen-Dürr.** 1992. Binding of the human E2F transcription factor to the retinoblastoma protein but not to cyclin A is abolished in HPV-16-immortalized cells. Oncogene **7:**1681–1686.
41. **Palombella, V. J., O. J. Rando, A. L. Goldberg, and T. Maniatis.** 1994. The ubiquitin-proteasome pathway is required for processing the NF-kappa b1 precursor protein and the activation of NF-kappa b. Cell **78:**773–785.
42. **Pan, H. C., and A. E. Griep.** 1994. Altered cell cycle regulation in the lens of HPV-16 E6 or E7 transgenic mice: implications for tumor suppressor gene function in development. Genes Dev. **8:**1285–1299.
43. **Phelps, W. C., K. Münger, C. L. Yee, J. A. Barnes, and P. M. Howley.** 1992. Structure-function analysis of the human papillomavirus E7 oncoprotein. J. Virol. **66:**2418–2427.
44. **Scheffner, M., K. Münger, J. M. Huibregtse, and P. M. Howley.** 1992. Targeted degradation of the retinoblastoma protein by human papillomavirus E7-E6 fusion proteins. EMBO J. **11:**2425–2431.
45. **Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine, and P. M. Howley.** 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell **63:**1129–1136.
46. **Schlegel, R., W. C. Phelps, Y.-L. Zhang, and M. Barbosa.** 1988. Quantitative keratinocyte assay detects two biological activities of human papillomavirus DNA and identifies viral types associated with cervical carcinoma. EMBO J. **7:**3181–3187.
47. **Slebos, R. J. C., M. H. Lee, B. S. Plunkett, T. D. Kessis, B. O. Williams, T. Jacks, L. Hedrick, M. B. Kastan, and K. R. Cho.** 1994. p53-dependent G_1 arrest involves pRB-related proteins and is disrupted by the human papillomavirus 16 E7 oncoprotein. Proc. Natl. Acad. Sci. USA **91:**5320–5324.
48. **Stubdal, H., J. Zalvide, and J. A. DeCaprio.** 1996. Simian virus 40 large T antigen alters the phosphorylation state of the RB-related proteins p130 and p107. J. Virol. **70:**2781–2788.
49. **Tommasino, M., J. P. Adamczewski, F. Carlotti, C. F. Barth, R. Manetti, M. Contorni, F. Cavalieri, T. Hunt, and L. Crawford.** 1993. HPV16 E7 protein associates with the protein kinase p33CDK2 and cyclin A. Oncogene **8:**195–202.
50. **Vousden, K. H., B. Vojtesek, C. Fisher, and D. Lane.** 1993. HPV-16 E7 or adenovirus E1A can overcome the growth arrest of cells immortalized with a temperature-sensitive p53. Oncogene **8:**1697–1702.
51. **Wazer, D. E., X. L. Liu, Q. Chu, Q. Gao, and V. Band.** 1995. Immortalization

of distinct human mammary epithelial cell types by human papilloma virus 16 E6 or E7. Proc. Natl. Acad. Sci. USA **92:**3687–3691.
52. **Weintraub, S. J., C. A. Prater, and D. C. Dean.** 1992. Retinoblastoma protein switches the E2F site from positive to negative element. Nature **358:**259–261.
53. **Werness, B. A., A. J. Levine, and P. M. Howley.** 1990. Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science **248:**76–79.
54. **White, A. E., E. M. Livanos, and T. D. Tlsty.** 1994. Differential disruption of genomic integrity and cell cycle regulation in normal human fibroblasts by the HPV oncoproteins. Genes Dev. **8:**666–677.
55. **Wu, E. W., K. E. Clemens, D. V. Heck, and K. Münger.** 1993. The human papillomavirus E7 oncoprotein and the cellular transcription factor E2F bind to separate sites on the retinoblastoma tumor suppressor protein. J. Virol. **67:**2402–2407.
56. **Yeager, T., W. Stadler, C. Belair, J. Puthenveettil, O. Olopade, and C. Reznikoff.** 1995. Increased p16 level correlate with pRb alterations in human uroepithelial cells. Cancer Res. **55:**493–497.
57. **zur Hausen, H.** 1991. Human papillomaviruses in the pathogenesis of anogenital cancer. Virology **184:**9–13.
58. **Zwerschke, W., S. Joswig, and P. Jansen Durr.** 1996. Identification of domains required for transcriptional activation and protein dimerization in the human papillomavirus type-16 E7 protein. Oncogene **12:**213–220.