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Abrogation of Growth Arrest Signals by Human Papillomavirus Type 16 E7 Is Mediated by Sequences Required for Transformation

G. WILLIAM DEMERS,† ERIK ESPLING, JO BETH HARRY, BETH G. ETSCHEID, AND DENISE A. GALLOWAY*

Program in Cancer Biology, Fred Hutchinson Cancer Research Center, Seattle, Washington 98040

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Cells arrest in the G_1 or G_0 phase of the cell cycle in response to a variety of negative growth signals that induce arrest by different molecular pathways. The ability of human papillomavirus (HPV) oncogenes to bypass these signals and allow cells to progress into the S phase probably contributes to the neoplastic potential of the virus. The E7 protein of HPV-16 was able to disrupt the response of epithelial cells to three different negative growth arrest signals: quiescence imposed upon suprabasal epithelial cells, G_1 arrest induced by DNA damage, and inhibition of DNA synthesis caused by treatment with transforming growth factor β. The same set of mutated E7 proteins was able to abrogate all three growth arrest signals. Mutant proteins that failed to abrogate growth arrest signals were transformation deficient and included E7 proteins that bound retinoblastoma protein in vitro. In contrast, HPV-16 E6 was able to bypass only DNA damage-induced G_1 arrest, not suprabasal quiescence or transforming growth factor β-induced arrest. The E6 and E7 proteins from the low-risk virus HPV-6 were not able to bypass any of the growth arrest signals.

Human papillomavirus (HPV) has been identified as an etiologic agent in anogenital cancers (63), yet only a particular subset of HPVs that infect the anogenital tract, such as types 16 and 18, are associated with a high risk of malignant conversion, whereas types such as 6 and 11 are almost always associated with benign proliferation of epithelial cells. The behavior of these viral types in vitro parallels the relationship observed in vivo, in that only the high-risk types efficiently transform rodent cell lines or immortalize primary rodent or human epithelial cells. E6 and E7 are the two viral proteins responsible for this behavior, based on the finding that E6 and E7 are invariably retained and expressed in tumors (51) and the genes encoding these two proteins are sufficient to immortalize cells in culture (28, 40).

E6 and E7 have many biological activities that may contribute to their ability to transform cells, but a key feature is the ability to inactivate cellular tumor suppressors. HPV-16 E6 (16E6) binds to p53 (59) in concert with a cellular ubiquitin-conjugating enzyme, E6AP, and targets p53 for degradation (49). Although the ability of HPV-6 E6 to bind p53 in vitro is controversial (9, 22, 59) it is clear that 6E6 does not target p53 for degradation, and the intracellular level of p53 in cells expressing 6E6 is unaffected (22). Sedman et al. (53) found that mutant p53 could cooperate with 16E7 to immortalize human keratinocytes, suggesting that the inactivation of p53 might be the sole function of E6 in immortalization; however, mutant p53 could not substitute for E6 in the transformation of rodent fibroblasts. Using mutants of 16E6 that resulted in degradation of p53 but failed to immortalize human embryonic kidney cells in cooperation with 16E7, Nakagawa et al. (43) concluded that degradation of p53 was necessary but not sufficient for E6-mediated immortalization.

The E7 proteins of several HPVs bind to the retinoblastoma (Rb) tumor suppressor, p105Rb (16), and other Rb family members such as p107 and p130 (11) through a well-defined motif, LxCxE. HPV-16 E7 binds Rb much more strongly both in vitro (41) and in vivo (23) than does HPV-6 E7, and the transformation activity parallels Rb-binding activity. Interestingly, HPV-1 E7 binds Rb with high affinity, although it does not transform cells (50). In that study, 1E7 was not able to transactivate an E2F-responsive promoter, suggesting that although 1E7 bound Rb, it was not able to inactivate the tumor suppressor activity of Rb, i.e., sequestering E2F transcription factor (6). Several studies have shown that mutated 16E7 proteins that were unable to bind to Rb were transformation deficient (3, 17, 29, 46, 56, 57), although one study has shown that immortalization of keratinocytes might not involve Rb binding (33). Although Rb binding was required for transformation, a number of mutated E7 constructs were fully competent to bind Rb but were still transformation deficient (3, 46). Residues in conserved region 1 of E1a have been implicated in the release of E2F from Rb (32); however, the CR1 domain of HPV-16 E7 does not appear to disrupt the Rb-E2F interaction (31, 55). Alternatively, some other function of E7 in addition to Rb binding may be necessary for transformation. Mutations in the amino-terminal region of both E1a and E7 also result in transformation-deficient oncoproteins. This region of E1a can bind to p300 (18), a CREB-related transcription factor (2). However, no p300-binding activity has been identified for E7. Furthermore, E1a constructs mutated in the p300-binding domain can complement E1a proteins mutated in the Rb-binding domain, whereas HPV-16 E7 constructs mutated in the Rb-binding domain could not complement E1a proteins mutated in the p300-binding domain (12). Additionally, mutations affecting the zinc fingers found in the carboxy-terminal end of E1a and E7 have both proved to be transformation deficient.

The ability of viral oncogenes to allow cells to continue to proliferate in the presence of negative growth arrest signals probably contributes to the neoplastic potential of the virus. We (13, 22) and others (30, 34, 54) have previously shown that E6 and E7 can bypass a DNA damage-induced G_1 arrest. In that case, arrest in G_1 is probably mediated by the induction of p53, which transcriptionally activates the expression of a cyclin-dependent kinase (cdk) inhibitor (CKI), p21 (19, 35). The

* Corresponding author. Mailing address: Program in Cancer Biology, Fred Hutchinson Cancer Research Center, 1124 Columbia St., Rm. C1-105, Seattle, WA 98040. Phone: (206) 667-4500. Fax: (206) 667-5815. Electronic mail address: dgallowa@fhcrc.org.

† Present address: Canji, Inc., San Diego, Calif.

ability of HPV-16 E6 to bypass a DNA damage-induced arrest was clearly linked to the ability to eliminate p53 (22), yet paradoxically, E7, which elevates the levels of p53 protein by a posttranscriptional mechanism (14), also bypassed G_1 arrest (13).

In fact many extrinsic growth inhibitory signals such as serum starvation, contact inhibition, DNA damage, and transforming growth factor β (TGF-β) induce the expression or activity of various CKIs that inhibit cyclin D- or cyclin E-associated complexes from phosphorylating Rb and other regulators of the G_1/S transition, resulting in arrest in the G_1 phase of the cell cycle. Several CKI family members have been implicated in inhibiting cdk activity in response to TGF-β. p27 was identified by its association with cyclin E-cdk2 complexes after TGF-β exposure (48), and p15 is upregulated by TGF-β exposure and inhibits cyclin D-cdk4 complexes (27). p21 is also transcriptionally activated by TGF-β but in a p53-independent manner (10). Expression of viral oncogenes simian virus 40 T antigen, adenovirus E1a, and HPV E7 have been shown to overcome TGF-β inhibition. In some studies (36, 47) the Rb binding site was shown to be necessary, while in other studies (1, 39) mutants of E1A that failed to bind Rb but retained p300-binding activity were at least partially resistant to TGF-β.

Immunohistochemical staining has suggested that differentiation of cervical or other stratified epithelia is accompanied by an induction of Rb protein (8). The suprabasal cells stained strongly for Rb, whereas basal cells had undetectable levels of Rb. Interestingly, p21 was also transcriptionally induced independently of p53 in differentiating keratinocytes (38). p21 induction was blocked by adenovirus E1a and a mutant of E1a that failed to bind Rb but retained the ability to bind p300. Overexpression of p300 suppressed the effect of E1a.

This study delves further into the ability of HPV oncogenes to bypass negative growth signals. The observation that the same set of mutated HPV-16 E7 proteins was able to interfere with a variety of negative growth signals suggests that E7 abrogates arrest by a common mechanism regardless of the inhibitory stimuli. HPV-16 E6 was able to abrogate only growth arrest that involved the p53 pathway. The inability of HPV-6 oncogenes to bypass growth-inhibitory signals probably reflects weak or more restricted interactions with cellular regulatory proteins.

MATERIALS AND METHODS

Cloning of mutated E7 genes into pLXSN. pBS16E7 contained HPV-16 coordinates 505 to 875 cloned into the *Hin*cII and *Pst*I sites of pBS(−). pBS16E7-x was created by replacing the *Hin*dIII site of pBS16E7 with a *Xho*I linker. Mutated E7 constructs (kindly supplied by Karen Vousden and Karl Munger) were digested with *Nsi*I and *Nco*I (566 to 863) and ligated to the *Nsi*I and *Nco*I fragment of pBS16E7-x to replace the wild-type sequences, except for pMo16E7-H2P, which was cleaved with and cloned into the *Pvu*II and *Pst*I (553 to 879) sites. The E7 constructs were cloned into pLXSN (37) by *Eco*RI and *Xho*I digestion of the pBS16E7-x mutant derivatives and insertion into similarly cleaved pLXSN. The entire E7 region was sequenced in the pLXSN constructs to confirm the mutations. Retroviruses were generated as described previously (25).

Expression of E7 protein. Total-cell lysates (20 μg) were run on sodium dodecyl sulfate (SDS)–15% polyacrylamide gels and transferred to polyvinylidene difluoride membrane. Western blots (immunoblots) were done with either a 1:50 dilution of HPV-16 E7 monoclonal antibody (Triton Diagnostics, Alameda, Calif.) followed by a 1:35,000 dilution of rabbit anti-mouse immunoglobulin G horseradish peroxidase conjugate or a 1:1,000 dilution of HPV-16 E7 polyclonal antibody (20) followed by a 1:20,000 dilution of goat anti-rabbit −peroxidase. Detection was by chemiluminescence (Renaissance; DuPont NEN, Boston, Mass.).

Binding of E7 to Rb and p107 in vitro. HPV-16 E7 mutants were cloned into pBS as described above. pRb4.6 and pBSskII p107 were kindly provided by E. Harlow and M. Ewen, respectively. Unlabeled in vitro-translated E7 proteins and [^{35}S]cysteine/methionine (Express Protein Labeling Mix; Dupont)-labeled in vitro translated Rb or p107 (10 μl each) were incubated on ice with cell lysis buffer (0.1 M Tris-HCl [pH 8.0], 0.1 M NaCl, 1% Nonidet P-40) for 2.5 h. The reaction products were immunoprecipitated with rabbit polyclonal anti-16 E7 sera (20) and protein A-Sepharose beads (Pharmacia) that had been equilibrated with 0.1 M Tris-HCl (pH 8.0)–0.1 M NaCl–0.1% Nonidet P-40–0.25% gelatin. The beads were washed four times with cell lysis buffer, resuspended in 20 μl of 2× SDS sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE; 12% polyacrylamide) and fluorography.

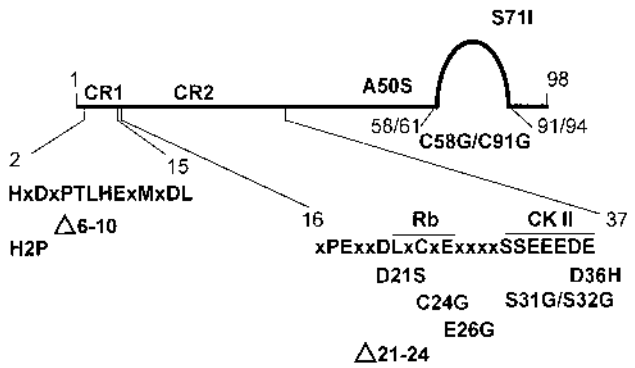

FIG. 1. Diagram of the HPV-16 E7 protein. The locations of regions of homology to the adenovirus E1a CR1 and CR2, including the motifs for binding to Rb and phosphorylation by casein kinase II (CK II), are indicated. The locations of mutations used in this study are shown.

Organotypic culture of human foreskin keratinocytes expressing mutated 16E7. Organotypic raft cultures were performed as described previously (4, 26). Briefly, 10^6 human keratinocytes that had been transduced with E7-expressing retroviruses were seeded onto a dermal equivalent of collagen and human foreskin fibroblasts. The cultures remained submerged for 7 days and were lifted to an air-liquid interface for 14 days. They were fed daily. The rafts were fixed in Methocarnoy solution (60% methanol, 30% chloroform, 10% acetic acid), embedded in paraffin, sectioned, and stained with hemotoxylin and eosin. Sections (5 μm) were deparaffinized and rehydrated. The sections were incubated with monoclonal antibody 19A2 reactive to proliferating-cell nuclear antigen (PCNA) (obtained from Allen Gown, University of Washington) and localized by the streptavidin-biotin immunoperoxidase method as previously described (24).

TGF-β inhibition of Mv1Lu cells expressing mutated 16E7. Mv1Lu cells (obtained from Bob Eisenman) were infected with LXSN retroviruses carrying mutated E7 constructs and selected with 2 mg of G418 per ml. Asynchronously growing cells were untreated or exposed to 3 ng of TGF-β per ml and 22 h later labeled with 0.01 mM bromodeoxyuridine (BrdU) for 2 h. The percentage of cells in S phase was determined from two-parameter analysis (propidium iodide versus anti-BrdU fluorescence) of flow cytometry results as previously described (13).

DNA damage of human mammary epithelial cells expressing mutated 16E7. Human mammary epithelial cells (a gift of S. A. Foster) were obtained from reduction mammaplasty tissue and were transduced with the E7 retroviruses. Induction of DNA damage and analysis of cell cycle parameters were performed as previously described (13). The cells were treated with 0.5 nM actinomycin D for 22 h and pulsed with 10 μM bromodeoxyuridine for 2 h. The cells were harvested, and the nuclei were stained with an anti-BrdU fluorescein isothiocyanate conjugate (Becton Dickinson) and propidium iodide. The nuclei were analyzed by FACScan (Becton Dickinson), and the percentage of cells in S phase was determined by using Reproman (True Facts Software).

RESULTS

E7 proteins bypass negative growth arrest signals. Epithelial cells can be arrested in G_1 or G_0 in response to a number of stimuli, including DNA damage and treatment with TGF-β, or by leaving the basal layer of the epithelium, although the mechanisms modulating the G_1 arrest appear to be different. To determine whether E7 can interfere with a variety of growth arrest signals suggesting a common mechanism, the E7 proteins from high- and low-risk papillomavirus types and a set of mutated HPV-16 E7 proteins were examined for their ability to bypass three growth-inhibitory signals in three types of epithelial cells.

A collection of plasmids encoding mutated E7 genes was generously provided by Karen Vousden, National Cancer Institute, Frederick, Md. (17), and Karl Munger, Harvard Med-

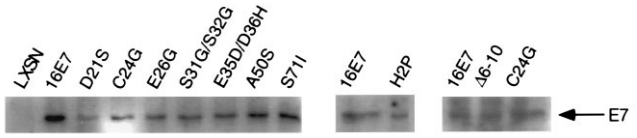

FIG. 2. Expression of the mutated E7 proteins. Whole-cell lysates were prepared from HFK transduced with retroviruses expressing vector alone (LXSN), HPV-16 E7, or the designated E7 mutant proteins. The proteins were resolved on 15% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The E7 proteins were detected with monoclonal antibody (left panel) or polyclonal rabbit sera (right panels) to E7, and visualized by chemiluminescence.

ical School (46) (Fig. 1). H2P and Δ6–10 are located in CR1; Δ21–24, C24G, and E26G alter the Rb-binding motif in CR2; D21S is located outside the Rb-binding motif within CR2; S31G/S32G eliminated phosphorylation by casein kinase II whereas D36H in the acidic recognition site for casein kinase II within CR2 did not alter phosphorylation; C58G/C91G eliminated the zinc finger in the carboxy-terminal domain whereas A50S and S71I, both within the carboxy-terminal domain, did not affect the zinc finger. To introduce the mutated E7 proteins efficiently into epithelial cells, recombinant amphotropic retroviruses expressing HPV-6 E7 and HPV-16 E7 (25) and the set of mutated HPV-16 E7 proteins were constructed. Following infection of primary human foreskin epithelium and selection in G418, expression of the E7 proteins was confirmed by Western blotting with a monoclonal antibody or polyclonal rabbit sera (25) (Fig. 2).

The epithelial cells were placed in organotypic culture and examined after 2 weeks. This method of culture induces cells to differentiate and form a stratified epithelial layer in which only basal cells proliferate. Cells transduced with the LXSN vector had an ordered epithelial layer with a characteristic pattern of differentiation in the parabasal and upper layers of the epithelium (Fig. 3). In contrast, cells expressing 16E7 had a disordered epithelium with proliferative cells throughout the epithelial layer (Fig. 3), as has been observed previously (4, 26). The mutated E7 proteins that were able to disrupt suprabasal quiescence similar to the wild-type 16E7 protein include D21S, D36H, A50S, and S71I (Fig. 3A; Table 1). The mutated E7 proteins that failed to disrupt suprabasal quiescence similar to the vector include H2P, Δ6–10, Δ21–24, C24G, E26G, and C58G/C91G. S31G/S32G was intermediate in phenotype, showing some proliferative cells above the basal layer. HPV-6 E7 was similar to the vector, with only a rare proliferative cell within the epithelial layer. The proliferative appearance of the cells was confirmed by staining with PCNA (Fig. 3B). HPV-16 E7 and those mutated E7 proteins that disrupted suprabasal quiescence showed cells throughout the thickness of the epithelium that stained positively for PCNA, whereas in mutants such as Δ21–24 and other E7 proteins that failed to disrupt suprabasal quiescence, PCNA-positive cells were restricted to the basal layer.

We previously showed that both E6 and E7 of HPV-16 but not HPV-6 were able to bypass DNA damage-induced growth arrest of human keratinocytes (13). The ability of E6 to abrogate the growth arrest signal correlated with the ability to target p53 for degradation (22); however, the mechanism by which E7 bypassed DNA damaged-induced G_1 arrest was unclear, since the cells retained high levels of p53. To investigate the mechanism more fully, human mammary epithelial cells were infected with the E7-expressing retroviruses. The cells were treated with low doses of actinomycin D to induce strand breaks, labeled with BrdU to identify cells that enter the S phase, and analyzed by fluorescence-activated cell sorter analysis (FACS) (Fig. 4; Table 1). Cells transduced with LXSN, HPV-6 E7, or the 16E7 mutants H2P, Δ6–10, Δ21–24, C24G, E26G, or C58G/C91G failed to enter S phase (85 to 98% inhibition), whereas 16 E7 or D21S, A50S, and S71I entered S phase as well as untreated cells did. Expression of the S31G/S32G E7 protein resulted in inhibited entry into S phase at approximately 60% of the level of cells that were not treated with actinomycin D.

Expression of viral oncogenes simian virus 40 T antigen, adenovirus E1a, and HPV E7 has been shown to overcome TGF-β inhibition. An established mink lung epithelial cell line, Mv1Lu, was chosen to examine the ability of the HPV oncogenes to confer resistance, because the mechanism of TGF-β inhibition has been studied extensively in this cell line and it has been used to examine resistance conferred by other viral oncogenes (48, 52). Mv1Lu cells were infected with the E7-expressing retroviruses, treated with TGF-β, labeled with BrdU, and analyzed for the appearance of cells in S phase as above (Fig. 4; Table 1). TGF-β inhibited the progression into S phase of cells expressing LXSN, HPV-6 E7, or the 16E7 mutants H2P, Δ6–10, Δ21–24, C24G, E26G, or C58G/C91G by 80 to 90%. The S31G/S32G E7 protein inhibited entry into S phase by 60%, and cells expressing HPV-16 E7, D21S, D36H, A50S, or S71I showed little inhibition of progression into S phase compared with untreated cells (10 to 30%).

The ability of E7 to bypass growth arrest signals correlates with transforming activity. The HPV-16 E7 protein and a set of mutated 16E7 proteins were able to bypass three distinct growth arrest signals, suprabasal quiescence, DNA damage, and TGF-β treatment, that probably involve different molecular pathways to arrest cells in G_1. The ability of E7 mutants to bind to Rb had been examined previously (11, 41, 46), yet those results did not correlate well with the ability to bypass growth arrest signals. To confirm the results, plasmids expressing the E7 proteins were translated in vitro and tested for their ability to bind to in vitro-translated Rb or p107 protein. Δ21–24, C24G, and E26G failed to bind to Rb in vitro, and Δ21–24 and C24G failed to bind p107; all the other mutated HPV-16 E7 proteins bound Rb and p107 approximately as well as the wild type did (Fig. 5; Table 1). All of the HPV-16 E7 proteins that were able to abrogate the growth arrest signals bound to Rb. Of the mutated E7 proteins that failed to abrogate growth arrest, Δ21–24, C24G, and E26G also failed to bind appreciably to Rb, although E26G retained the ability to bind p107. However, H2P, Δ6–10, and C58G/C91G all bound Rb at levels that could not be distinguished from wild-type 16E7, yet they failed to abrogate growth arrest.

The ability of mutated E7 genes to transform rodent cells has been examined extensively with consistent results (3, 17, 29, 46, 57). Deletion or mutation of the Rb-binding motif as in Δ21–24, C24G, and E26G eliminated the ability of E7 to transform cells. However, mutation of other residues, which do not affect Rb binding, such as H2P, Δ6–10, and C58G/C91G, also eliminated the ability of E7 to transform. These data have been interpreted to indicate that inactivation of Rb is necessary but not sufficient for transformation. The mutants D21S, D36H, A50S, and S71I transformed rodent cells with efficiencies similar to the wild type. While some studies (46) have shown that altering the casein kinase II site had no effect on transformation, other studies (21, 56) showed that the transformation efficiency was reduced when serines 31 and 32 were mutated. The ability of E7 to bypass growth arrest correlated with the ability to transform cells and suggested that functions in addition to inactivation of Rb are required.

Inability of HPV-16 E6 and HPV-6 oncoproteins to bypass growth arrest signals. Critical to the ability of the cell to

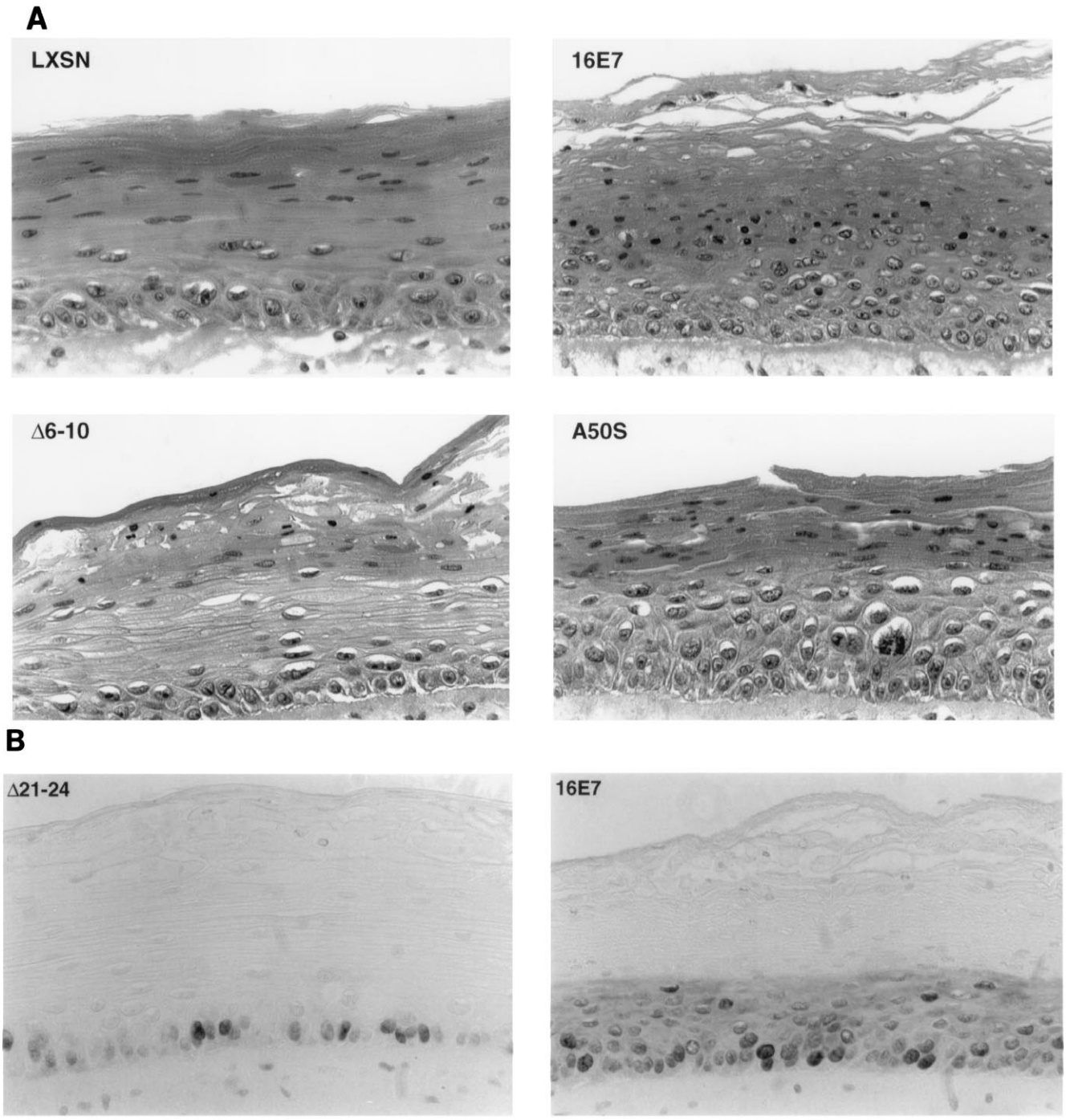

FIG. 3. Organotypic raft cultures of cells expressing HPV 16 E7 proteins. (A) Human foreskin epithelial cells expressing the vector, HPV-16 E7, 16E7 Δ6–10, and 16E7 A50S were grown in organotypic culture, fixed, sectioned, and stained with hemotoxylin and eosin. (B) Sections were stained with a monoclonal antibody to PCNA.

respond to DNA damage is an intact p53 gene (35). The expression of HPV-16 E6 prevented G_1 arrest following irradiation or treatment with DNA-damaging agents (13, 34); using mutants of E6, we demonstrated that the ability of E6 to abrogate a DNA damage-induced G_1 arrest was linked to the elimination of p53 protein (22). To determine whether E6, like E7, could bypass other growth arrest signals, the effects of E6 on overcoming TGF-β treatment or suprabasal quiescence were examined. Figure 6 demonstrates that E6-expressing Mv1Lu cells were as sensitive to TGF-β treatment as were vector-containing cells, i.e., 80% inhibition of entry into S phase by 0.1 ng of TGF-β per ml and >90% inhibition by 0.3 ng/ml, whereas E7 expression allowed 50% entry into S phase at 0.1 to 3 ng/ml. We had previously observed that HPV-16 E6 expression alone did not alter the proliferation of epithelial cells grown in organotypic culture (26) and have confirmed those results (data not shown).

A number of studies have shown that the oncogenes from

TABLE 1. Growth arrest characteristics of HPV oncoproteins

Construct[a]	Growth arrest behavior as measured by:					
	Transformation[b]	Suprabasal quiescence	DNA damage	TGF-β inhibition	Rb binding	p107 binding
LXSN	−	−	−	−	−	−
wt HPV-6 E7	−	−	−	−	±	±
wt HPV-16 E7	+	+	+	+	+	+
H2P	−	−	−	−	+	+
Δ6–10	−	−	−	−	+	+
Δ21–24	−	−	−	−	−	−
D21S	+	+	+	+	+	+
C24G	−	−	−	−	−	−
E26G	−	−	−	−	−	+
S31G/S32G	±	±	±	±	+	+
D36H	+	+	NT[c]	+	+	NT
A50S	+	+	+	+	+	NT
C58G/C91G	−	−	−	−	+	+
S71I	+	+	+	+	+	+

[a] wt, wild type.
[b] Results taken from references 17 and 46.
[c] NT, not tested.

the low-risk viruses do not transform cells in culture. We have previously demonstrated that neither E6 nor E7 from HPV-6 is able to bypass a DNA damage-induced G_1 arrest (13, 22). Figure 4 confirms these data with results obtained with 6E7 and also demonstrates that 6E7 cannot bypass a TGF-β-induced arrest. This result is consistent with an earlier report that showed that HPV-6 E7 was not able to abrogate the TGF-β-mediated repression of the c-*myc* promoter (42). As with 16E6 (Fig. 6), HPV-6 E6-expressing cells were sensitive to TGF-β (data not shown). Our previous data also showed that neither E6 nor E7 from HPV-6 could efficiently stimulate the proliferation of suprabasal epithelial cells, although occasional cells throughout the epithelium expressed PCNA (26). Taken together, these data indicate that whereas HPV-16 E7 can abrogate three distinct growth arrest signals, HPV-16 E6 can bypass only growth arrest signals mediated by induction of p53. Additionally, although HPV-6 E7 can bind to Rb and transactivate E2F-responsive promoters, it is not able to bypass growth arrest signals, consistent with its lack of transforming activity.

DISCUSSION

The focus of this study was to examine the ability of HPV oncogenes to escape negative growth signals. We chose to examine three separate means to inhibit the entry of epithelial cells into S phase: suprabasal quiescence, DNA damage, and exposure to TGF-β. In each case, the molecular mechanisms of

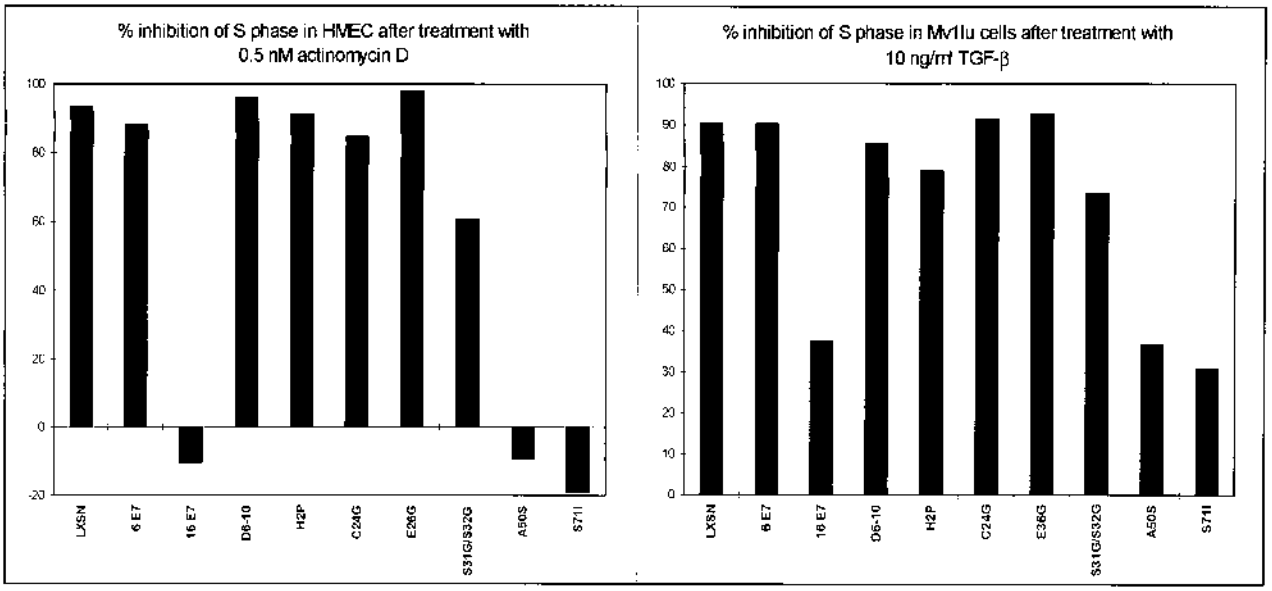

FIG. 4. HPV-16 E7 bypasses G_1 arrest induced by DNA damage or TGF-β. (A) Human mammary epithelial cells expressing E7 proteins were exposed to 0.5 nM actinomycin D for 22 h and pulsed with BrdU for 2 h, and nuclei were prepared and labeled with antibody to BrdU and propidium iodide. The percentage of cells was determined after FACS analysis. (B) Mv1Lu cells expressing the E7 retroviruses were exposed to 10 ng of TGF-β per ml, and the percentage of cells in S phase was determined as for DNA damage.

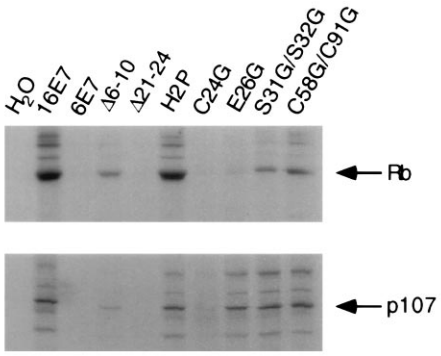

FIG. 5. In vitro binding of E7 to Rb and p107. Rabbit reticulocyte lysates primed with H_2O or HPV E7 constructs were incubated with [^{35}S]cysteine/methionine-labeled Rb or p107 rabbit reticulocyte lysates. After immunoprecipitation with an anti-16E7 antiserum, samples were run on SDS-PAGE (12% polyacrylamide).

arrest are distinct but related; each inducer of arrest causes an increase in a CKI family member and therefore the inhibition of cdk activity, resulting in the inability to phosphorylate Rb and other targets required for the transition from G_1 to S. The ability of a cell to induce p53 and consequently p21 in response to DNA damage is critical for arrest in G_1 (5, 35). Elimination of p53 by HPV-16 E6 leads to low levels of p21 and efficient bypass of the DNA damage-induced G_1 checkpoint; HPV-16 E7 bypasses that checkpoint by a distinct method, independent of p53. TGF-β treatment has been reported to induce at least three CKIs, p27, p15, and p21, although the relative importance of each CKI to growth arrest has not been evaluated. p53 is not induced by TGF-β (reference 10 and our unpublished data), and the HPV-16 E6 protein cannot abrogate the inhibitory effects of TGF-β. Although the molecular mechanism that imposes a G_1 arrest on epithelial cells as they leave the basal layer is not fully known, both Rb and p21 have been implicated. Induction of p21 was not dependent on p53 (38, 45), and elimination of p53 by 16E6 did not disrupt suprabasal quiescence. It should be noted that epithelial cell differentiation is apparently normal in mice lacking p21 (15), suggesting that other CKIs or other mechanisms contribute to suprabasal quiescence. Thus, the HPV-16 E6 oncogene was able to bypass only negative growth signals that involved the p53 pathway. The inability of HPV-6 E6 to bypass DNA damage-induced growth arrest suggests that 6E6 is not able to inactivate p53.

The fact that HPV-16 E7 and the same set of mutated E7 proteins responded identically in all three growth arrest assays suggests that the general details of the mechanism may be similar. If the G_1 arrest induced by TGF-β is mediated by p27 and/or p15 whereas p21 is responsible for DNA damage-induced G_1 arrest, then E7 would be capable of abrogating G_1 arrest mediated by distinct CKI family members, suggesting that the mechanism of abrogation is not targeted at the individual CKI family members. The finding that the ability of the E7 mutants to bypass growth-inhibitory signals corresponded to the ability to transform cells suggests that these two processes may be related.

HPV-6 E7 was not able to abrogate any of the three growth arrest signals tested. We had previously observed that 6E7 had a very limited ability to induce proliferation in organotypic cultures (26) or enter S phase after DNA damage (13). Similarly, Watanabe et al. (58) had reported a reduced ability of 6E7 to induce DNA synthesis in rat 3Y1 cells. In contrast, Cheng et al. (7) showed that the HPV-11 E7 gene induced proliferation in organotypic culture as efficiently as HPV-16 E7 did. Although retrovirus vectors were used to introduce the genes into epithelial cells in both studies, different promoters were used to drive the expression of E7. A more likely explanation is that the HPV-11 E7 protein may be more like the high-risk E7 proteins. For example, Munger et al. (41) examined the binding of various E7 proteins to Rb and found that 11E7 was 4- to 6-fold weaker than 16E7 whereas 6E7 was ~20-fold weaker. The in vivo association of 6E7 with Rb was also much weaker than that of 16E7 (23).

The precise mechanism by which HPV-16 E7 bypasses growth arrest signals is not fully understood. Mutants of E7 that failed to bind to Rb, Δ21–24, C24G, and E26G were not able to bypass growth arrest signals, consistent with earlier data indicating that Rb-negative mutants of simian virus 40 T antigen or E1a were sensitive to TGF-β (47). These data suggest that the inactivation of Rb and consequent release of E2F transcription factor is required for progression into S phase. Consistent with this observation is the recent demonstration that overexpression of the E2F1 gene was sufficient to overcome TGF-β inhibition of Mv1Lu cells (52). However, these data do not agree with two reports in which E1a proteins that did not bind Rb, either fully or partially, conferred TGF-β resistance. Our data suggested that binding to Rb was necessary but not sufficient for E7 to disrupt cellular growth control. Transformation assays involving mutated E7 constructs have shown the same requirement (3, 17, 29, 46). In a model sug-

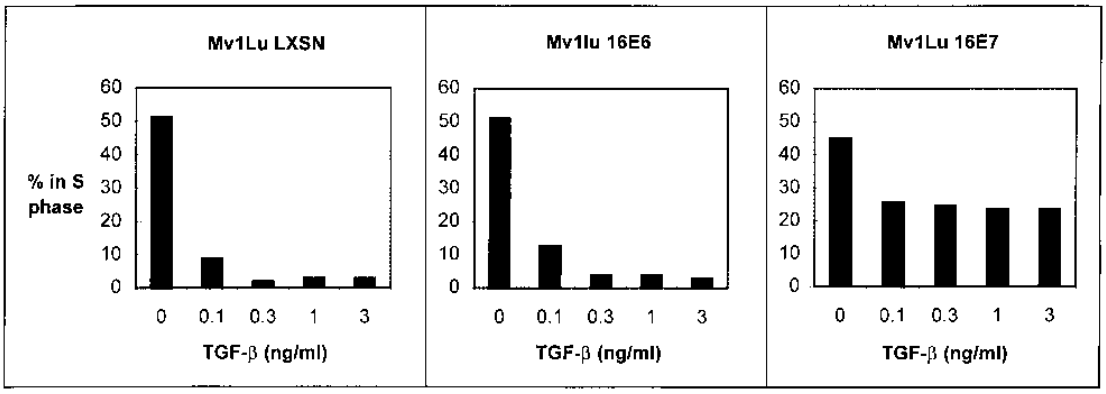

FIG. 6. Sensitivity of HPV-16 E6 and E7 to TGF-β. Mv1Lu cells transduced with LXSN, HPV-16 E6, or HPV-16 E7 were treated with TGF-β at the indicated concentrations for 22 h and then subjected to a 4-h labeling with BrdU. The percentage of cells in S phase was determined by FACS analysis.

gested by Ikeda and Nevins (32), the CR2 domain of adenovirus E1a was required for initial Rb association and CR1 was necessary to release E2F from the complex. Regions of E7 homologous to the CR1 domain of adenovirus E1a, including the region deleted in Δ6–10 and the mutation H2P, were not able to bypass growth arrest signals and were transformation deficient. Previous studies report conflicting results of whether various CR1 mutations in E7 are affected in their ability to transactivate an E2F-responsive promoter (3, 17, 46, 57). Unlike E1a, the CR1 domain of HPV-16 E7 played no role in the dissociation of E2F-Rb complexes (31, 55). Mutations in the zinc finger of E7 have not been extensively studied for their effects on E2F-mediated transcription; however, elimination of one or both Cys-X-X-Cys motifs severely reduced or eliminated the ability of E7 to displace E2F (31, 55). Thus, it is unlikely that all of the effects of E7 in bypassing growth arrest signals and in mediating transformation could be due to the ability of E7 to liberate E2F.

The observation that the CKI p21 is associated with cyclin-cdk complexes from proliferating cells that retain kinase activity lead to the realization that changing active complexes to inactive complexes is accomplished by changing the stoichiometry of p21 in the complexes (62). The regulation of G_1 arrest in normal cells is dependent on the ratio of CKIs to cyclin-cdk complexes. This paradigm can be used as a means to regulate G_1 arrest in E7-expressing cells, in which bypassing G_1 arrest can be accomplished by increasing the ratio of cyclin-cdk complexes to CKIs. The recent demonstration that cyclin E is one of the E2F-responsive genes (44) and that cyclin E levels are elevated in E7-expressing cells (36a, 61) suggests that increasing the number of cyclin E-cdk2 complexes could titrate out the CKIs induced by negative growth stimuli.

Alternatively, inactivation of other cellular proteins such as the CREB-related p300 protein might be involved in the ability of E7 to bypass G_1 arrest. It is intriguing that the induction of p21 in differentiating keratinocytes was mediated by p300 (38). Finally, E7 might have additional activities that prevent CKIs from inhibiting cyclin kinase complexes. The composition of cyclin-cdk complexes is not altered in proliferating human fibroblasts expressing E7 (60), indicating that E7 does not prevent p21 from associating with the complex; however, the composition of the complexes after a growth arrest signal was not examined. It will be important to determine the precise mechanism by which HPV-16 E7 bypasses growth arrest signals.

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