Role of the Retinoblastoma Pathway in Senescence Triggered by Repression of the Human Papillomavirus E7 Protein in Cervical Carcinoma Cells

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ABSTRACT

Repression of the endogenous human papillomavirus (HPV) type 18 E7 gene in HeLa cervical carcinoma cells by the bovine papillomavirus E2 transcription factor activates the retinoblastoma (Rb) pathway and induces cells to undergo senescence. To determine whether activation of the Rb pathway is responsible for senescence in response to HPV18 E7 repression, we tested the ability of wild-type and mutant E7 proteins to affect the activity of the Rb pathway and to modulate senescence in these cells. Enforced expression of the wild-type HPV16 E7 protein prevented Rb activation in response to E2 expression and impaired senescence. Importantly, there was an absolute correlation between the ability of mutant E7 proteins to inactivate the Rb pathway and to inhibit senescence in HeLa cells. Similar results were obtained in HT-3 cervical carcinoma cells. These results provide strong genetic evidence that activation of the Rb pathway is required for senescence in response to E7 repression. Hence, continuous neutralization of the Rb pathway by the E7 protein is required to maintain the proliferation of cervical carcinoma cells. Similarly, our results indicate that activation of the Rb pathway can prevent apoptosis induced by repression of the HPV18 E6 gene in HeLa cells.

INTRODUCTION

Cervical carcinoma, a leading cause of cancer mortality in women worldwide, is initiated by infection with high-risk human papillomaviruses (HPV), usually HPV16 or HPV18. Integration of the viral DNA into the infected host cell genome often disrupts the gene that encodes the E2 protein, a viral transcription factor that can repress expression of the HPV E6 and E7 oncoproteins (1). The high-risk HPV E6 protein binds to p53 and stimulates its ubiquitin-directed degradation (2–4). The E6 protein also stimulates the activity of telomerase, which maintains the ends of chromosomes (5).

A conserved LXCXE motif in the E7 protein is absolutely required for E7/Rb binding and accelerated proteasome-mediated degradation of Rb family members, and an NH2-terminal segment of the E7 protein is required for Rb degradation but not for Rb binding (11, 16–22). The E7 protein binds to numerous other cellular proteins, but the physiological consequences of these interactions are largely unknown (6).

The E6 and E7 proteins have profound effects on cells. Primary human cells cannot be passaged indefinitely in culture, rather they undergo replicative senescence, an irreversible growth-arrested state characterized by a constellation of features including flattened morphology, elevated autofluorescence, and increased senescence-associated β-galactosidase (SAβ-gal) activity (23). Genetic studies in primary keratinocytes revealed that activation of telomerase and inactivation of the Rb and p53 pathways are required to bypass senescence and immortalize cells (24–26). Expression of the high-risk E6 and E7 genes also allows primary keratinocytes to bypass senescence, but HPV-immortalized cells are not tumorigenic, implying that additional events are required for carcinogenic progression (2, 6).

Continuous expression of the high-risk HPV E6 and E7 proteins is required to maintain the proliferative state of cervical cancer cells. Antisense and RNA interference-mediated repression of HPV gene expression in cervical carcinoma cell lines typically results in a severe and partial inhibition of proliferation (e.g., Refs. 27 and 28). More dramatic effects are induced by the papillomavirus E2 proteins. Introduction of the bovine papillomavirus (BPV) E2 gene into cervical carcinoma cell lines or HPV-immortalized keratinocytes represses expression of the resident HPV genomes, resulting in activation of the p53 and Rb pathways and inhibition of telomerase activity (29–36). E2-mediated repression of E6 and E7 expression can cause close to 99% inhibition of cellular DNA synthesis within 2 days, loss of colony forming ability, and the rapid acquisition of a senescent phenotype by virtually every cell in the population, suggesting that the E6 and E7 proteins actively prevent the execution of a senescence program (31, 32, 34, 37). BPV E2-induced senescence requires repression of the HPV oncogenes and does not occur in cells devoid of HPV DNA (32, 38, 39).

Expression of the E2 protein in HeLa cells engineered to constitutively express the HPV16 E6 protein represses the endogenous HPV18 E7 protein, resulting in activation of the Rb pathway and senescence, even though continued expression of the HPV16 E6 protein prevents activation of the p53 pathway (39). Furthermore, E2-mediated repression of HPV30 expression induces senescence in HT-3 cervical carcinoma cells, even though these cells do not contain transactivation-competent p53 (31, 40). Taken together, these findings imply that repression of E7 is sufficient to induce a robust, p53-independent senescence response.

The cellular signaling pathways responsible for senescence induced by E7 repression have not been defined. In this report, we tested the hypothesis that Rb activation is required for induced senescence when the E7 gene is repressed. An exogenous HPV16 E7 gene inhibits senescence caused by E2-mediated repression of the endogenous HPV18 E7 gene in HeLa cells (39). Here, we tested the ability of a panel of E7 mutants to inhibit senescence triggered by E7 repression, with the rationale that senescence would not be inhibited by E7 mutants defective for Rb inactivation if senescence required Rb sig-
RESULTS

Expression of Mutant HPV16 E7 Proteins in HeLa Cells. The E2 protein represses the endogenous HPV18 E6 and E7 genes in HeLa cells and induces greater than 95% of the cells to undergo growth arrest and senescence. However, when the wild-type HPV16 E7 protein is constitutively expressed in these cells, approximately one-third of the cells fail to undergo senescence in response to the E2 protein. These nonsenescent cells continue to proliferate and undergo a wave of p53-dependent apoptosis 5–7 days after E2 expression (39, 43). The ability of the E7 protein to provide partial protection against senescence and to induce apoptosis in response to E2 expression provides an assay for the activity of E7 mutants. We used recombinant retroviruses to introduce wild-type and mutant HPV16 E7 genes into HeLa cervical carcinoma cells. The following HPV16 E7 mutations were tested: deletion Δ6–10, located in the NH₂-terminal segment of the E7 protein, which does not interfere with binding to Rb family members but does interfere with their degradation; deletion Δ21–24, which blocks Rb binding and degradation by removing the LXCXE motif; S31G/S32G, which is located near the LXCXE motif and eliminates E7 phosphorylation by casein kinase II; and A50S, a phenotypically silent mutation located near the middle of the E7 protein (44).

The empty retrovirus vector (LXSN) and vectors containing the wild-type and mutant HPV16 E7 genes were used to infect HeLa/SEN2 cells, and individual G418-resistant colonies were expanded to generate clonal cell lines. Western blotting confirmed the absence of HPV16 E7 protein from the HeLa/LXSN cells and demonstrated that the other cell lines expressed similar levels of the wild-type or mutant HPV16 E7 protein (Fig. 1). The E2 protein was then acutely introduced into these cells by infection with a recombinant SV40-based viral vector expressing the BPV E2 protein, which acts as a transcriptional repressor of the endogenous HPV18 E6 and E7 genes in HeLa cells but not the introduced HPV16 E7 gene driven by the retroviral long terminal repeat. Thus, after E2 expression, the exogenous wild-type or mutant HPV16 E7 protein is the only HPV oncogene product expressed in the cells, providing an assay for the activity of the E7 protein.

Biochemical Analysis of E7 Mutant Cell Lines after Repression of the Endogenous HPV18 E6 and E7 Oncogenes. The biochemical and physiological responses of these cells to E2 expression were determined. As expected, 2 days after E2 infection, expression of the endogenous HPV18 E6-E7 oncogenes was markedly repressed in all of the cell lines (data not shown). The E2 protein caused a marked increase in the level of p53 in all of the cell lines due to repression of the endogenous HPV18 E6 gene and loss of E6-induced destabilization of p53 (Fig. 2, bottom panel). Furthermore, the E2 protein caused a decrease in the level of hyperphosphorylated p105Rb in all cell lines (Fig. 2, top panel), presumably at least in part as a consequence of p53-mediated induction of the cdk inhibitor, p21.

In the absence of the E2 protein, all of the cell lines displayed naling. Our results provide strong evidence that activation of the Rb pathway is required for senescence in response to E7 repress.
similar patterns of p105Rb expression and phosphorylation, in which the slowly migrating, hyperphosphorylated form is the most prominent species (Fig. 2, top panel). Less p105Rb was present in the cells transduced with the wild-type E7 gene than in the other cell lines. Expression of the E2 protein in HeLa/LXSN cells, which express no endogenous E7 protein, caused a marked increase in the level of the hypophosphorylated, active form of p105Rb due to repression of endogenous HPV18 E7 in these cells and loss of E7-mediated p105Rb destabilization. Constitutive expression of the wild-type HPV16 E7 protein or E7A50S prevented E2-mediated induction of hypophosphorylated p105Rb, because these E7 proteins bind this form of p105Rb and induce its accelerated degradation. In contrast, in cells expressing E7A6–10 or E7A21–24, the E2 protein induced the expression of hypophosphorylated p105Rb to a level comparable with that in cells not expressing an exogenous E7 protein, consistent with the inability of these mutants to stimulate Rb degradation. Constitutive expression of E7A50S prevented E2-mediated induction of hypophosphorylated p105Rb, because these E7 proteins bind this form of p105Rb and induce its accelerated degradation. In contrast, in cells expressing E7A6–10 or E7A21–24, the E2 protein induced the expression of hypophosphorylated p105Rb to a level comparable with that in cells not expressing an exogenous E7 protein, consistent with the inability of these mutants to stimulate Rb degradation. Constitutive expression of E7A50S resulted in a slight increase in the level of the hypophosphorylated form of Rb after E2 infection. We also assessed the expression of cyclin A, the product of an E2F-responsive gene (Fig. 2, middle panel). The E2 protein repressed expression of cyclin A in HeLa/LXSN cells, indicating that the Rb pathway was activated, but not in HeLa/16E7 and HeLa/16E7A50S cells. Cyclin A expression was also inhibited by the E2 protein in the HeLa/16E7A6–10 and HeLa/16E7A21–24 cells, whereas HeLa/16E7A50S cells displayed slight repression of cyclin A expression. Thus, E7A6–10 and E7A21–24 are defective in their ability to inactivate the Rb pathway in cervical carcinoma cells, and repression of the endogenous E7 protein in cells expressing these mutants activates the Rb pathway.

Effect of E7 Mutants on the Cellular Response to the E2 Protein. To assess the effect of the E7 mutants on cell proliferation, we measured incorporation of tritiated thymidine into DNA 2 days after E2 infection or mock infection (Fig. 3). As reported previously, the E2 protein dramatically inhibited DNA synthesis in HeLa/LXSN cells due to repression of the endogenous HPV18 E6 and E7 genes (39). Constitutive expression of the wild-type HPV16 E7 protein provided substantial protection against E2-mediated growth inhibition, with E2-infected HeLa/16E7 cells displaying approximately 30% DNA synthesis of uninfected cells. Notably, E2 expression caused dramatic inhibition of DNA synthesis in HeLa/16E7A6–10 and HeLa/16E7A21–24 cells, similar to the level of inhibition displayed by cells without constitutive E7 expression. In contrast, infected HeLa/16E7A50S cells displayed similar levels of DNA synthesis as cells expressing the wild-type E7 protein, and HeLa/16E7A50S cells displayed intermediate levels of DNA synthesis. Similar results were obtained with multiple independent isolates of each genotype. Thus, the wild-type E7 protein and E7A50S, both of which prevent activation of the Rb pathway, conferred substantial protection against E2-induced growth arrest, whereas the two mutants defective for Rb inactivation did not provide protection, and the mutant that allowed partial activation of the Rb pathway provided partial protection.

We also measured SAβ-gal expression (Fig. 4; data not shown). The colonies arising from mock-infected cells showed faint background staining (mock-infected HeLa/16E7A6–10 cells are shown as a representative example). After infection with the E2 virus, virtually all HeLa/LXSN cells displayed intense blue staining indicative of SAβ-gal activity and cellular senescence. In contrast, as reported previously (39), the E2-infected HeLa/16E7 cultures contained cells displaying a flattened morphology and SAβ-gal activity interspersed with numerous proliferating colonies that did not stain, demonstrating partial protection from senescence by the wild-type HPV16 E7 protein. Notably, expression of the E2 protein in HeLa/16E7A6–10 or HeLa/16E7A21–24 (data not shown) cells resulted in uniform high-level SAβ-gal activity, demonstrating that these E7 mutants did not inhibit senescence. E2-infected HeLa/16E7A50S (data not shown) and HeLa/16E7A50S cells displayed a mixture of proliferating SAβ-gal-negative cells and flat, nonproliferating SAβ-gal-positive cells.

Because HeLa cells constitutively expressing the wild-type HPV16 E7 protein undergo apoptosis as well as senescence upon repression of
the endogenous HPV18 oncogenes (39), we also measured apoptosis. Nonpermeabilized cells were stained with annexin V and propidium iodide 6 days after E2 infection or mock infection and analyzed by flow cytometry (Fig. 5A). In this analysis, early apoptotic cells bind annexin but exclude propidium iodide and are visualized in the lower right quadrant of a two-dimensional plot. Few E2-infected HeLa/LXSN cells displayed annexin binding, although these cells showed increased fluorescence in the lower left quadrant, indicative of increased autofluorescence due to senescence. In contrast, E2-infected HeLa/16E7 cells displayed a greater than 10-fold increase in the number of apoptotic cells compared with mock-infected cells. Strikingly, E2 expression did not induce elevated apoptosis in cells expressing either of the Rb-defective mutants, E7Δ5–10 or E7Δ21–24, but these cells displayed an autofluorescence shift characteristic of senescing cells (39). Thus, cells expressing E7 mutants unable to inactivate the Rb pathway underwent senescence but not apoptosis, as did cells without constitutive E7 expression. E2-infected HeLa/16E7ASOS and HeLa/16E7Δ31GΔ32G cells displayed intermediate levels of apoptosis. The results of multiple independent annexin-binding experiments are summarized in Fig. 5B.

The experiments described above indicated that E7 proteins able to inactivate the Rb pathway inhibited senescence and stimulated apoptosis in HeLa cells after repression of the endogenous HPV18 E6 and E7 genes. In contrast, cells expressing either of the E7 mutants defective for Rb inactivation efficiently senesced and failed to undergo apoptosis, whereas a mutant displaying partial Rb-inactivating activity was intermediate in blocking growth inhibition. Taken together, these results indicate that inactivation of the Rb pathway inhibits E2-mediated senescence and permits apoptosis. We infer that activation of the Rb pathway is required for senescence and inhibition of apoptosis after E7 repression.

Biochemical Effect of Rb-Deficient E7 Mutants in Cells Constitutively Expressing the HPV16 E6 Protein. The interpretation of the results described in the previous sections is complicated by the complex phenotype induced by the E2 protein in cells constitutively expressing the wild-type HPV16 E7 protein. HeLa cells engineered to constitutively express the HPV16 E6 protein display simpler responses to the E2 protein. In these cells, E2 expression efficiently induces senescence, but constitutive expression of the HPV16 E7 protein together with the HPV16 E6 protein prevents E2-induced senescence, and the cells continue to proliferate without undergoing apoptosis (39). Therefore, E7 mutants defective for Rb binding and degradation were introduced into cells constitutively expressing the HPV16 E6 protein, and the response of these cells to the E2 protein was determined.

HeLa/16E6H cells, which constitutively express the HPV16 E6 protein, were infected with the LXSN vector or retroviruses expressing the wild-type E7 protein, E7Δ5–10, or E7Δ21–24, and cell lines were established from individual colonies resistant to both G418 and hygromycin. The E2 protein repressed expression of the endogenous HPV18 E6 and E7 genes in these cells (data not shown). In the
presence of the E2 protein, these cell lines expressed similar levels of the wild-type and mutant HPV16 E7 proteins, which were higher than expression in the absence of the E2 protein (Fig. 6). Because high-risk HPV E6 proteins can act as transcriptional repressors of retroviral long terminal repeats (45), E2-mediated induction of the E7 protein may be a consequence of repression of the endogenous HPV18 E6 gene.

The effect of the E2 protein was determined in two independent cell lines expressing each of these retroviruses. Because all of these cell lines constitutively expressed the HPV16 E6 protein, p53 was not induced by E2 expression (Fig. 7, bottom panel). In control HeLa/16E6H-LXSN cells, the E2 protein caused a marked induction of the hypophosphorylated form of p105Rb, due to repression of the endogenous HPV18 E7 protein. In addition, the level of the hyperphosphorylated form was decreased, due to Rb-mediated repression of E2F-responsive genes required for maximal cdk activity, such as cyclin A and cdc25A. The E2 protein had no effect on the expression or phosphorylation of p105Rb in cells expressing the wild-type HPV16 E7 protein. Strikingly, hypophosphorylated p105Rb was eliminated in cells expressing the E7 mutants defective for Rb binding and degradation. Induction of hypophosphorylated p105Rb was more dramatic in cells expressing E7Δ21–24, which is unable to bind or degrade Rb family members, than in cells expressing E7Δ6–10, which can bind but not degrade p105Rb. Similarly, the E2 protein repressed cyclin A expression in cells with no added E7 protein or expressing the E7 mutants unable to bind or degrade Rb, whereas cyclin A expression persisted in cells expressing the wild-type HPV16 E7 protein. Thus, as expected, the E2 protein did not activate the Rb pathway in cells constitutively expressing the wild-type HPV16 E7 protein, but it did activate the pathway in cells expressing E7 mutants defective in their interaction with Rb family members.

Phenotypic Effect of Rb-Deficient E7 Mutants in HeLa Cells Constitutively Expressing the HPV16 E6 Protein. To assess the effect of the E7 mutants on cell proliferation after expression of the E2 protein in cells constitutively expressing the E6 protein, incorporation of tritiated thymidine into DNA was measured in several cell lines of each genotype (Fig. 8). The E2 protein dramatically inhibited DNA synthesis in cells constitutively expressing HPV16 E6, whereas constitutive expression of the wild-type E7 protein in these cells largely abrogated E2-induced growth inhibition. E2 expression caused dramatic inhibition of DNA synthesis in multiple clones of HeLa/16E6–16E7Δ6–10 and HeLa/16E6–16E7Δ21–24 cells also. Thus, unlike the wild-type E7 protein, the two E7 mutants defective for Rb inactivation were unable to prevent growth arrest induced by repression of the endogenous HPV18 E7 protein in these cells.

SAβ-gal expression was also examined in cell lines that were mock-infected or infected with the E2 virus (Fig. 9). In all cases, mock-infected cell lines generated colonies that showed faint background staining, as did E2-infected cells constitutively expressing the wild-type E7 protein. After infection with the E2 virus, virtually all of the HeLa/H16E6H-LXSN cells displayed a flat, loosely packed morphology and blue staining, demonstrating SAβ-gal activity and senescence. Expression of the E2 protein also induced SAβ-gal activity and senescent morphology in cells coexpressing HPV16 E6 and
either E7Δ6–10 or E7Δ21–24, demonstrating that these mutants did not protect against senescence.

Flow cytometry was used to measure autofluorescence in cells expressing the HPV16 E6 protein and either the wild-type E7 protein or the E7 mutants defective for Rb inactivation (Fig. 10). As expected, the entire population of E2-infected HeLa/16E6H-LXSN cells displayed a dramatic increase in autofluorescence, consistent with the efficient induction of senescence, whereas constitutive expression of the wild-type E7 protein blocked this increase. E2 expression also caused markedly increased autofluorescence in cells coexpressing HPV16 E6 and either E7 mutant. Similar results were obtained with multiple independent cell lines of each genotype. Thus, cells expressing Rb-deficient E7 mutants, like cells not expressing an exogenous E7 protein, efficiently underwent senescence in response to E2 expression.

Modulation of Senescence by Adenovirus E1A Protein. We also isolated clonal HeLa cell lines coexpressing HPV16 E6 and similar levels of the adenovirus 5 13S E1A protein or a mutant E1A protein containing a point mutation that disrupted the Rb-binding motif. The wild-type E1A protein markedly inhibited E2-mediated senescence, but the mutant did not (data not shown). Thus, as was the case for the HPV16 E7 protein, the E1A protein inhibited E2-induced senescence in HeLa cells in an Rb-dependent fashion, confirming that Rb activation was required for senescence induced by E7 repression in HeLa cells.

Role of the Rb Pathway in HT-3 Cells. We also tested the requirement for the Rb pathway in HT-3 cervical carcinoma cells, which express HPV30 E6 and E7 genes. E2 expression activates the Rb pathway and induces senescence in these cells (31, 35, 40), although this process is less efficient than in HeLa cells. Because HT-3 cells harbor only transactivation-defective p53, the E2 protein does not activate the p53 pathway in these cells. HT-3 cells were infected with the empty retrovirus vector or retroviruses expressing wild-type HPV16 E7 or E7Δ21–24, and G418-resistant colonies were pooled to establish polyclonal cell lines. DNA synthesis was measured after mock infection or E2 infection. As shown in Fig. 11, the E2 protein inhibited DNA synthesis in HT-3/LXSN cells to a significant extent, consistent with the induction of senescence in most of the cells. Constitutive expression of the wild-type HPV16 E7 protein largely eliminated the E2-induced reduction in DNA synthesis in HT-3/16E7 cells, demonstrating that senescence required E7 repression in HT-3 cells. Strikingly, the E7Δ21–24 mutant did not prevent E2-induced senescence in HT-3/16E7Δ21–24 cells. Similarly, other measures of senescence, i.e., cell enlargement and flattening, SAβ-gal activity and increased autofluorescence were inhibited by the wild-type E7 protein but not by E7Δ21–24 (data not shown). Thus, senescence induced by E7 repression in HT-3 cells required activation of the Rb pathway, as it did in HeLa cells. This result also implies that E6 repression did not activate a p53-independent senescence pathway in HT-3 cells.

DISCUSSION

Repression of the endogenous HPV E7 oncogene triggers senescence in cervical carcinoma cell lines. However, because the high-risk E7 protein binds to numerous cellular proteins, the biochemical pathways responsible for this senescence response have not been established. To determine whether activation of the Rb pathway was required for senescence in response to E7 repression, we tested the ability of the wild-type and mutant E7 proteins to affect the activity of the Rb pathway and modulate induced senescence.

Expression of the wild-type HPV16 E7 protein inhibited senescence caused by E2-mediated repression of the endogenous HPV18 E7 gene in two HeLa cell genetic backgrounds. When the endogenous HPV18 E6 and E7 genes were both repressed in HeLa cells, the exogenous E7 protein provided partial protection against senescence, and some of the cells underwent apoptosis. When the HPV18 E7 gene was repressed but HPV16 E6 expression was maintained in HeLa/16E6H cells, the exogenous E7 protein prevented senescence. In both cell types, two independent E7 mutants unable to inactivate the Rb pathway failed to inhibit senescence. Similarly, HeLa cell senescence was inhibited by the wild-type E1A protein but not an E1A mutant unable to bind Rb. An E7 mutant that retained the ability to inactivate the Rb pathway provided the same level of protection against senescence in HeLa cells as did the wild-type E7 protein, and an E7 mutant with an intermediate effect on Rb activity partially blocked growth inhibition. Senescence induced by E7 repression in HT-3 cells was also blocked by the wild-type HPV16 E7 protein but not by an Rb-binding-defective mutant. These experiments provide compelling genetic evidence that senescence initiated by E7 repression in HeLa cervical carcinoma cells requires activation of the Rb pathway. Rb signaling is also required for ras-induced senescence in human fibroblasts (46) and for replicative senescence (24–26, 47, 48) upon serial
cell passage. Furthermore, introduction of an exogenous Rb gene can induce senescence in human cancer cells (49, 50).

Wells et al. (37) also found that senescence triggered by combined repression of the HPV18 E6 and E7 genes in HeLa cells was partially inhibited by the wild-type HPV16 E7 gene but not by an E7 mutant unable to bind Rb. However, there are important differences between these earlier experiments and those reported here. Importantly, we conducted biochemical analysis to establish the activity of the Rb pathway in HeLa cells expressing the wild-type and mutant exogenous E7 proteins. In addition, as well as examining senescence when both E6 and E7 were repressed, we also examined the requirement for Rb signaling in senescence triggered by repression of E7 in the absence of E6 repression and in HT-3 cells in the absence of p53 signaling. Because E6 repression initiates p53-dependent signaling in HeLa cells, which can also induce senescence in the absence of Rb activity (39, 43), interpretation of experiments involving repression of both E6 and E7 is difficult. Thus, it is important to separately analyze the activities of the E6 and E7 proteins to dissect their influence on cellular physiology.

We also explored the role of the Rb pathway in apoptosis induced by E6 repression. When the endogenous HPV18 E6 protein is repressed in cells expressing the wild-type HPV16 E7 protein, a fraction of the cells undergo apoptosis after a delay, a response that requires p53 activity (39, 43). We found here that E6 repression induced apoptosis only in cells expressing E7 proteins that inactivated the Rb pathway. Thus, active Rb signaling inhibited p53-dependent apoptosis in cervical carcinoma cells. In several additional cell systems, Rb activity inhibits E7-mediated apoptosis (17, 51, 52).

Previous mutational and biochemical studies identified two features of the wild-type E7 protein required for full inactivation of Rb, the LXCXE Rb-binding motif and an NH2-terminal degradation motif. The inability of the E7Δ6–10 mutant to prevent senescence initiated by repression of the HPV18 E7 gene indicates that Rb binding is not sufficient to inactivate the Rb senescence pathway in HeLa cells and that Rb degradation is also required. The S13G/S32G double mutant, which lacks the casein kinase II phosphorylation sites, partially protects against both Rb activation and the senescence response, implying that this mutant interferes with senescence by impairing the Rb pathway. Other investigators showed that phosphorylation of the CKII sites does not modulate Rb binding in vitro, but that mutations at these sites can impair Rb degradation (17, 44, 53). Similarly, our experiments show that mutations at these sites can result in inefficient Rb degradation and impaired repression of E2F-responsive S-phase genes such as cyclin A.

The available genetic analysis implies that E7 is a modular protein and that it is possible to assign individual activities to specific, fairly well-defined segments of the protein. For example, biochemical and gene transfer experiments demonstrated that NH2-terminal mutations that interfere with Rb degradation do not impair Rb binding, stimulation of S phase or apoptosis, E2F displacement in vitro, and transactivation of E2F-responsive genes; mutations at the CKII phosphorylation sites do not affect Rb binding at the adjacent LXCXE site but do affect Rb degradation and repression of p53 transcriptional activity; and p21 binding is separable from Rb inactivation (22, 44, 52–58).

In addition, a COOH-terminal activity of the E7 protein is required for abrogation of exogenous growth inhibition stimuli by neutralizing p21 activity (42, 55). Therefore, the inability of E7Δ6–10 or E7Δ21–24 to prevent senescence is unlikely to be due to E7 activities other than loss of Rb inactivation, although we note that the regions of the E7 protein required for Rb binding and degradation are also required for binding IRF-1 (59). Analysis of additional E7 mutants may reveal the existence of other E7 activities in addition to Rb inactivation that are required to prevent senescence.

In summary, both binding and degradation of the Rb proteins by the HPV E7 protein are essential for sustained proliferation of HeLa cervical carcinoma cells, and E7 repression triggers senescence at least in part by activating the Rb pathway in both HeLa and HT-3 cells. We previously showed that senescence initiated by E6 repression in HeLa cells required activation of the p53 pathway (43). Thus, HPV repression activates at least two pathways that can result in senescence, the Rb pathway when E7 is repressed and the p53 pathway when E6 is repressed. Our results also imply that Rb signaling can prevent the delayed, p53-dependent apoptosis elicited by E6 repression. Future strategies to inhibit the growth of cervical cancer cells should include approaches that manipulate these dormant growth inhibitory pathways.

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