Adeno-Associated Virus Type 2 Increases Proteosome-Dependent Degradation of p21\textsuperscript{WAF1} in a Human Papillomavirus Type 31b-Positive Cervical Carcinoma Line

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Infection of the mucosal epithelium of the anogenital region by high-risk human papillomavirus (HPV) types is positively associated with the development and neoplastic progression of cervical carcinoma (12, 44, 83, 84). Specifically, high-risk HPV types 16, 18, 31, 33, and 45 are associated with over 95% of all cervical carcinoma (12, 44, 83, 84). Specifically, high-risk HPV associated with the development and neoplastic progression of cervical cancers (28, 81, 85). The tumorigenicity of HPV has been attributed to the E6 and E7 oncogenes, which interfere with the functions of cellular tumor suppressors p53 (61) and pRb (14, 42), respectively. Both oncoproteins are involved in the release of the G1 cell cycle block in differentiating keratinocytes, thus deregulating key cell cycle checkpoints and promoting viral DNA replication (25). On the other hand, infection with the adeno-associated virus type 2 (AAV2), a helper-dependent human parvovirus, may have a negative effect against the development of cervical cancer (46). Seroprevalence studies have demonstrated that women with HPV-associated cervical carcinoma have antibodies to AAV less frequently compared to matched controls (27, 46, 68, 71). More recent epidemiological studies have also suggested that the presence of AAV DNA in cervical tissue (19) and AAV2 seropositivity (68) are associated with a reduced risk of developing high-grade cervical squamous intraepithelial lesions. Thus, AAV has been proposed to antagonist the development of HPV-related carcinoma (45, 62).

The antiproliferative and tumor-suppressive properties of AAV have been suggested to result from its ability to target oncogenic viral functions. Historically, AAV2 has been described as a helper-dependent parvovirus, as it requires the presence of another helper virus, such as adenovirus (15) or herpesvirus (10, 20), for its own gene expression and infectious virion synthesis (9). In turn, AAV mediates suppression of its helper virus DNA replication. Infection with AAV2 was also shown to inhibit growth of cell lines derived from cervical lesions (72, 77). The tumor-suppressive activities of AAV2 against HPV were first demonstrated by its ability to inhibit bovine papillomavirus (33), as well as HPV-induced transformation in culture (32, 34). We have recently demonstrated that AAV2 inhibits HPV type 31b (HPV31b) DNA replication in the differentiation-dependent raft culture system, concomitant with active AAV2 replication as well as infectious AAV2 progeny virion production (50). We also reported that HPV provides enhancer/helper functions for AAV2 replication (50), as our laboratory was also the first to demonstrate that AAV2 is capable of autonomous replication and progeny virion formation in the differentiation-dependent raft culture system, in the absence of any known helper viruses (52). In parallel, in vivo evidence suggested the possibility of an AAV-HPV interaction based on the finding that DNA of both viruses coexists in the same cervical epithelial lesion (76), as well as in penile condylomatous lesions (3). Studies based on data derived from patient tissue samples suggested that AAV2 infects the human genital tract, specifically the cervix (73, 74, 78), which is also a target for HPV infections (82, 84). Since both HPV (83) and AAV (24, 73, 76) are anogenital viruses, it is possible that the two viruses interact in the cervical epithelium, giving rise to potential interference of HPV-associated tumor development (57, 76).

In addition to targeting helper virus functions, AAV2 has...
also been shown to target host cellular factors. Although the molecular mechanisms underlying AAV’s ability to promote oncosuppression via this route are not well understood, a few studies point to cell cycle regulatory proteins as targets. AAV2 was shown to protect the p53 (6) and pRB (4) tumor suppressors from adenovirus-mediated degradation and subsequent oncogenic progression. Moreover, AAV2 was shown to negatively regulate transcription of E2F-1 as well as potentiating inhibitory protein-protein interactions between pRB and E2F-1 in adenovirus-infected cells (5). Another study pointed to the ability of AAV2 to selectively mediate pRB hypophosphorylation (60). Under conditions nonpermissive for AAV replication and morphogenesis, AAV2 infection of primary human fibroblasts was shown to upregulate expression of the p21WAF1 cyclin-dependent kinase (CDK) inhibitor and promote pRB hypophosphorylation, both factors associated with a G1-phase cell cycle block, decreasing cellular proliferation rates and growth arrest (31). Cumulatively, studies portraying the ability of AAV2 to target key cellular proteins suggest that deregulation of cell cycle checkpoints by oncogenic viruses may be antagonized by AAV2.

Studies of AAV2-mediated cell cycle regulation in HPV-infected cells have not been reported. Our laboratory has begun characterizing the molecular mechanism underlying AAV2-mediated oncosuppression by examining cell cycle modulation in HPV-positive cell lines. Our results demonstrate that AAV2 infection of HPV-positive cells results in targeting of the p21WAF1 protein for proteosome-mediated degradation. Our results support the idea that the initial stages of AAV2 superinfection of HPV-infected cells give rise to changes in cell cycle protein profiles mimicking an enhanced G1/S transition, with a simultaneous delay in S phase progression. In addition, we propose that one of the actions of AAV2 against HPV is via targeting of cell cycle proteins deregulated in a low-grade cervical lesion.

MATERIALS AND METHODS

**Viral stocks.** AAV2 stocks were prepared in our laboratory by utilizing the whole-cell lysis method, followed by purification using CsCl gradients and detergent. In addition, we propose that one of the actions of AAV2 targeting of the p21WAF1 protein for proteosome-mediated degradation that AAV2 infection of HPV-positive cells results in modulation in HPV-positive cell lines. Our results demonstrate that the ability of AAV2 to selectively mediate pRb hypophosphorylation was shown to protect the p53 (6) and pRB (4) tumor suppressors from adenovirus-mediated degradation.

**Studies of AAV2-mediated cell cycle regulation in HPV-infected cells have not been reported.** Our laboratory has begun characterizing the molecular mechanism underlying AAV2-mediated oncosuppression by examining cell cycle modulation in HPV-positive cell lines. Our results demonstrate that AAV2 infection of HPV-positive cells results in targeting of the p21WAF1 protein for proteosome-mediated degradation. Our results support the idea that the initial stages of AAV2 superinfection of HPV-infected cells give rise to changes in cell cycle protein profiles mimicking an enhanced G1/S transition, with a simultaneous delay in S phase progression. In addition, we propose that one of the actions of AAV2 against HPV is via targeting of cell cycle proteins deregulated in a low-grade cervical lesion.

**Protein concentrations were measured using the Peterson protein assay (Lowry method) as previously described (50). To determine expression of various proteins, 30 μg of whole-cell extract was used for detecting p21WAF1, p27KIP1, CDK2, and actin expression by utilizing Western blotting. For detecting p53 protein expression, 60 μg of the whole-cell extract was used.**

**Immunoprecipitation.** For immunoprecipitating protein complexes, cell extracts were prepared from cell pellets as follows. Each cell pellet consisting of 2 × 106 cells was resuspended in 600 μl buffer A (described above). Samples were transferred into a 1.5-ml microcentrifuge tube. To the suspension, 400 μg of ice-cold acid-washed glass beads (0.45 mm; Sigma) was added. Tubes were placed in a multibead holder and vortexed for 30 seconds at maximum power. Samples were then chilled on ice for 30 seconds. This cell breakage procedure was repeated a total of five times. The microcentrifuge tubes were placed on the rim of 13- by 100-mm precooled disposable glass tubes. An 18-gauge needle was used to puncture holes through the lid and bottom of the microcentrifuge tube. The piggy-back microcentrifuge tube and glass tube combination was centrifuged to attain a speed of 2,000 rpm and then stopped. The microcentrifuge tube was discarded. The eluted samples were transferred into a 1.5-ml microcentrifuge tube. Supernatants were then collected by centrifugation for 5 min at 4°C. Protein concentrations were measured using the Bradford assay.

**Immunoprecipitation buffer was prepared using the following composition:** 1% Triton X-100, 10 mM HEPES (pH 7.5), 2 mM EDTA, 50 mM NaF, 0.2 mM Na3VO4, 2 mM DTT, 200 mM NaCl, 20 μg/ml aprotinin, 0.5 μg/ml pepstatin, 0.5 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride. For each immunoprecipitation reaction mixture, a total of 200 μg of protein extract was used. The volume was made up to 1 ml with immunoprecipitation buffer supplemented with 2% nonfat dry milk. All samples were first preclarified using 5 μl of mouse anti-tyrosin kinase AE1/AE3 monoclonal antibody (Chemicon International) by incubating for 1 h at 4°C with rotation. To each sample, 50 μl of prescreened protein A-Sepharose beads (Amersham Biosciences) was added and incubated at 4°C for 30 min with rotation. Beads were removed by centrifugation at 13,000 rpm for 20 s. The supernatant was removed and added to a fresh tube. The preclarified lysates were then subjected to immunoprecipitation using polyclonal antibodies against CDK2 (sc-163), p21WAF1 (sc-397), and cyclin A (sc-751) (Santa Cruz Biotechnology), monoclonal antibodies against p27KIP1, cyclin E (sc-247) (Santa Cruz Biotechnology), and p53 (Oncogene). For immunoprecipitation with polyclonal antibodies, 3.5 μl of each antibody was used per reaction mixture. For immunoprecipitation with monoclonal antibodies, 5 μl of each antibody was used. After addition of antibody, all samples were incubated at 4°C for 1.5 h with rotation, followed by addition of 50 μl of prescreened protein A-Sepharose beads as the immune complex binding agent. Samples were further incubated at 4°C for 1 h with rotation. Beads were pelleted by centrifugation at 13,000 rpm for 20 s and then washed three times with 500 μl ice-cold immunoprecipitation buffer without milk supplementation. After the final wash the beads were resuspended in 60 μl of 1× sample loading buffer and boiled for 10 min in a water bath. A total of 10 μl of each sample was used for detecting respective proteins in the immunoprecipitated complexes utilizing Western blot analysis.

The medium was aspirated from the plates, and infections were carried out using AAV2 at a multiplicity of infection (MOI) of 100. The AAV2 stocks were diluted into 1 ml of E medium without serum and used for infection of cells. Mock infections were performed using 1 ml of E medium without serum. Plates were incubated at 37°C for 2 h with intermittent swirling. At the end of the incubation, the virus-containing medium was aspirated from the plates and replaced with 10 ml of fresh E medium supplemented with serum. Both mock-infected and AAV2-infected cell samples were trypsinized, inactivated with the addition of serum, pelleted, and stored at ~70°C until further manipulations. Samples of infected and uninfected cells, consisting of 2 × 106 cells per sample, were collected at 0, 2, 4, 6, 10, 18, and 24 h postinfection with AAV2.

**Preparation of whole-cell extracts.** Total protein extracts were prepared as follows. Frozen cell pellets consisting of 2 × 106 cells per sample as starting material were thawed on ice and resuspended in 250 μl buffer A (50 mM NaPO4, pH 7.2, 5 mM EDTA, 50 mM NaF, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 20 μg/ml aprotinin, 0.2 mM Na3VO4, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride). To this suspension, 250 μl buffer B was added (identical to buffer A but supplemented with 2% [wt/vol] sodium dodecyl sulfate [SDS]), and the suspension was boiled in a water bath for 8 min. While boiling, the samples were vortexed briefly every 2 min. Samples were transferred into a 1.5-ml microcentrifuge tube, and supernatants were collected by centrifugation for 5 min at room temperature. From each sample, a 500-μl aliquot of the supernatant was removed, and 125 μl of 5% [vol/vol] sucrose was added. Samples were placed into a multibead holder and vortexed for 30 seconds at maximum power. Samples were then chilled on ice for 30 seconds. This cell breakage procedure was repeated a total of five times. The microcentrifuge tubes were placed on the rim of 13- by 100-mm precooled disposable glass tubes. An 18-gauge needle was used to puncture holes through the lid and bottom of the microcentrifuge tube. The piggy-back microcentrifuge tube and glass tube combination was centrifuged to attain a speed of 2,000 rpm and then stopped. The microcentrifuge tube was discarded. The eluted samples were transferred into a 1.5-ml microcentrifuge tube. Supernatants were then collected by centrifugation for 5 min at 4°C. Protein concentrations were measured using the Peterson protein assay (Lowry method) as previously described (50).

**Immunoprecipitation reaction mixture, a total of 200 μg of protein extract was used. The volume was made up to 1 ml with immunoprecipitation buffer supplemented with 2% nonfat dry milk. All samples were first preclarified using 5 μl of mouse anti-tyrosin kinase AE1/AE3 monoclonal antibody.**
Western blot analysis. Protein extracts were applied to an SDS-polyacrylamide gel (acylamide/ bis-acylamide ratio, 30:0.8). Gel compositions for resolving various proteins were as follows: 12% gel to detect p21WAF1, 10% gel for p27KIP1 and CDK2, 8% gel for actin, 6% gel for Rb and cyclin A, and 7.5% gel to detect cyclin E, p53, and E2F. Proteins were transferred to a nitrocellulose membrane (Protran; Schleicher & Schuell). Membranes were incubated overnight with primary antibodies. The following dilutions of primary antibodies were used to detect respective proteins. Polyclonal antibodies against p21WAF1, p27KIP1, CDK2, cyclin A, and pRb were each used at a dilution of 1:2,000 to detect the respective proteins from immunoprecipitates as well as from whole-cell extracts. Monoclonal antibodies against cyclin E, E2F, and p53 were used at a dilution of 1:1,000 to detect the respective immunoprecipitated proteins in Western blots. Monoclonal antibody against actin (ICN clone C4) was used at a dilution of 1:5,000 to detect actin expression in whole-cell extracts. After being washed, the blots were incubated with anti-rabbit or anti-mouse horseradish peroxidase-linked secondary antibody (Amersham Life Science) as per the manufacturer’s instructions. The proteins were detected using the enhanced chemiluminescence method (Perkin-Elmer) as per the manufacturer’s instructions. In order to determine equal loading of total immunoprecipitated samples in the Western blots, identical blots were stained with GelCode Blue stain reagent (Pierce) after proteins were transferred onto nitrocellulose membranes.

Histone H1 kinase assay. The histone H1 kinase assay was performed as previously described (17). Briefly, immunoprecipitation of AAV2-infected and mock-infected CIN-612 9E, HFK, and HPV31:b monolayer samples was performed using polyclonal antibodies against CDK2 as described above. Immunoprecipitates were washed once with immunoprecipitation buffer (described above) and then twice with kinase reaction buffer (50 mM HEPES [pH 7.5], 10 mM MgCl2, 10 mM MnCl2, and 1 mM DTT). Kinase reactions were performed in a final volume of 20 µl consisting of kinase buffer supplemented with 20 µM ATP, 10 µCi of [γ-32P]ATP, and 1 µg of histone H1 (Roche) as substrate. Kinase assay mixtures were incubated at 30°C for 30 min. Reactions were stopped with the addition of loading buffer, and samples were boiled for 10 min. Reactions were resolved by electrophoresis on a 12% SDS-polyacrylamide gel and dried, followed by autoradiography.

Flow cytometric analysis of DNA content. Cells were prepared for analysis as described previously (38). Cells were harvested by trypsinization, washed with phosphate-buffered saline (PBS), fixed in 70% ethanol, and stored at −20°C. The fixed cells were washed in PBS and then resuspended in PBS containing 0.1% Triton X-100 (Sigma), 200 µg/ml of DNase-free RNase A (Boehringer Mannheim), and 100 µg/ml of propidium iodide (Sigma) for 30 min at room temperature. Flow cytometric analysis of 106 cells was carried out in a fluorescence-activated cell sorter (FACS), and the percentages of cells in the G1, S, and G2/M phases of the cell cycle were determined using the Cell Quest program of Becton Dickinson. Data were analyzed with the Mod Fit LT program.

RESULTS

AAV2 infection differentially regulates expression of the CDK inhibitor p21WAF1 in HPV31b-positive cells. It has been previously demonstrated that AAV2-mediated inhibition of cell cycle progression is associated with upregulated p21WAF1 protein expression in primary human fibroblasts (31). In normal cells, overexpression of p21WAF1 is associated with inhibition of two critical cell cycle checkpoints in G1 and G2, through both p53-dependent and -independent pathways (41). An increase in p21WAF1 protein levels is also indicative of increased binding to G1- and S-phase cyclin/CDK complexes, resulting in the inhibition of their kinase activity and consequent block in cell cycle progression (13, 29, 36). To investigate whether the p21WAF1 protein was also a target for AAV2 in HPV-infected cells, we used the CIN-612 9E cell line, which maintains episomal HPV31b genomes and is capable of producing infectious HPV31b virions upon differentiation in raft cultures (49, 51). These cells are derived from a preneoplastic cancer biopsy and maintain many of the characteristics of cells as they occur in vivo (8, 21, 58). As a control we used primary human foreskin keratinocytes for infection with AAV2. The use of keratinocytes is a relevant model, because keratinocytes are the natural hosts for both HPV and AAV2 and provide representative physiological comparisons of relevant cell cycle changes. Both CIN-612 9E and HFK cell lines were synchronized as described in Materials and Methods, when approximately two-thirds or more of the cells were in the G1 phase of the cell cycle (Fig. 1A and B). In our hands, this was the maximum level of synchronization we were able to achieve under the culture conditions used here. For comparison, FACS profiles of confluent CIN-612 9E and HFK cells used for cell synchronization are presented alongside the data (Fig. 1C and D) to portray the active cycling nature of the cells at the time of infection with AAV2 (Fig. 1A and B). We infected the synchronized cultures of CIN-612 9E cells as well as primary HFK keratinocyte cultures with AAV2 at an MOI of 100. Total protein extracts were prepared, followed by Western blot analysis of the expression profile of the p21WAF1 protein. The results of a representative Western blot analysis are shown in Fig. 2. Data from multiple analyses were combined and are graphically presented in Fig. 3. In CIN-612 9E cells, p21WAF1 protein levels were decreased in response to AAV2 infection compared to mock-infected controls (Fig. 2A and 3A). The pattern of decreased p21WAF1 expression in response to AAV2 in HPV-infected cells was reproducible in multiple experiments (Fig. 3). In contrast, AAV2 infection of HFK lines resulted in
increased \textit{p21WAF1} expression in AAV2-infected cultures compared to mock-infected controls (Fig. 2B and 3B). Upregulation of \textit{p21WAF1} in HFK cells at 18 and 24 h post-AAV2 infection underwent a 3.5- to 4.0-fold change (Fig. 3B). As would be expected, in mock-infected cell samples \textit{p21WAF1} protein levels changed according to the progression of the cell cycle as a function of time. The differential effects of AAV2 on \textit{p21WAF1} levels in the two cell lines used cannot be explained by differences in the ability of AAV2 to infect the two cell lines. Southern blot assays performed to compare amounts of incoming AAV2 DNA at the 2-h time point in both HPV-positive and primary keratinocyte lines were shown to be similar (results not shown). AAV2-mediated upregulation of \textit{p21WAF1} protein levels in primary HFK cultures (Fig. 2B) was similar to the AAV2-induced increase of \textit{p21WAF1} in primary human fibroblasts (31). However, the abundance of \textit{p21WAF1} was decreased following AAV2 infection in HPV31b-positive keratinocytes. These results suggest that the presence of the HPV genome and/or its associated gene products determines the outcome of \textit{p21WAF1} as a target for AAV2.

To determine the specificity of \textit{p21WAF1} as a target for AAV2, we also detected the expression of the \textit{p27KIP1} CDK inhibitor, which is functionally similar to \textit{p21WAF1} in that both inhibit kinase activity of cyclin E/CDK2 complexes (13, 36). In contrast to changes in \textit{p21WAF1} expression, relative abundance of \textit{p27KIP1} protein levels remained unchanged upon AAV2 infection in both HPV31b-positive and primary HFK cell lines (Fig. 2). We also determined expression of actin, which also remained unchanged (Fig. 2). Our results demonstrate the specificity of \textit{p21WAF1} as a target for AAV2 in these cells.

![Figure 2](http://jvi.asm.org/)  
**FIG. 2.** AAV2 downregulates \textit{p21WAF1} protein levels in HPV-positive keratinocytes. CIN-612 9E (A) and HFK (B) cell lines were infected with AAV2 at an MOI of 100, trypsinized, and pelleted at the stated time points within a 24-h period. Whole-cell extracts were prepared, and 30 μg of total protein sample was resolved on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and probed with \textit{p21WAF1} polyclonal antibodies as indicated. To detect \textit{p27KIP1} protein expression, 30 μg of whole-cell extract was resolved on a 10% SDS-polyacrylamide gel and probed with a polyclonal antibody against \textit{p27KIP1} as described for \textit{p21WAF1}. To detect actin expression, 30 μg of whole-cell extract was resolved on an 8% SDS-polyacrylamide gel and probed with a monoclonal antibody against actin as described for \textit{p21WAF1}.

![Figure 3](http://jvi.asm.org/)  
**FIG. 3.** Densitometric analysis of immunoprecipitated \textit{p21WAF1} protein levels in AAV2-infected and uninfected CIN-612 9E and HFK cell lines. CIN-612 9E (A) and HFK (B) cell lines were infected with AAV2 as described in Materials and Methods. Cell extracts were prepared, followed by immunoprecipitation with the antibody against \textit{p21WAF1}. Immunoprecipitates were resolved on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose, and Western blotting was performed with the polyclonal antibody against \textit{p21WAF1}. Densitometric analysis of bands corresponding to the immunoprecipitated \textit{p21WAF1} was performed. Data presented are the means of three experiments. At each time point, immunoprecipitated \textit{p21WAF1} protein levels in mock-infected samples were normalized to 1.
**Effects of AAV2 superinfection on p21WAF1/cyclin E/CDK2 complexes.** Normally, the p21WAF1 CDK inhibitor exists as a member of complexes with G1/S cyclins and CDK subunits (29) as well as with PCNA (75). In general, interaction of p21WAF1 with the G1/S cyclin E/CDK2 complex inhibits kinase activity of CDK2, which delays pRb phosphorylation, subsequent G1/S transition, and S phase progression (16, 55). We wanted to determine whether changes in p21WAF1 protein levels had affected its interaction with G1 cyclin E/CDK2 complexes and, consequently, their activities. As before, we infected CIN-612 9E and HFK cultures with AAV2, followed by immunoprecipitation of p21WAF1 from uninfected and AAV2-infected samples at time points within a 24-h period (Fig. 4A and B). Each immunoprecipitation reaction was initiated using 200 μg of protein extract prepared as described in Materials and Methods. Immunoprecipitated p21WAF1 samples were analyzed for the presence of coprecipitated CDK2 as well as cyclin E by Western blotting using polyclonal antibodies against these proteins. In HPV31b-positive cells, the amounts of CDK2 and cyclin E bound to p21WAF1 were decreased proportionally to the total p21WAF1 levels in the immunoprecipitate (Fig. 4A). This conclusion is supported by the observations that total CDK2 levels remained unchanged and cyclin E was increased upon AAV2 infection in the HPV31b-positive cell line (Fig. 4C and E). An identical blot was analyzed for equal loading of total protein amounts in immunoprecipitated samples by staining the total protein using the GelCode Blue stain reagent (Fig. 4A and B).

We also analyzed p21WAF1/cyclin E/CDK2 complexes in the HFK cell cultures infected with AAV2. In HFK cells, p21WAF1 protein levels were upregulated following AAV2 infection, whereas in uninfected HFK cells, p21WAF1 levels were lower, representative of normal progression of the cell cycle (Fig. 4B). In contrast to our results with HPV31b-positive cells, the level of p21WAF1 complexes from mock-infected and AAV2-infected HFK cultures could not be correlated with stoichiometric changes in binding of p21WAF1 with CDK2 (Fig. 4B). However, in HFK cells differential binding of p21WAF1 to the two
phosphorylated forms of CDK2 was evident. At 2 h postinfection, p21WAF1 in AAV2-infected HFK samples could be found bound predominantly to the active, hypophosphorylated (33-kDa) form of CDK2 (Fig. 4B, lane 3). In contrast, p21WAF1 from mock-infected HFK cultures at 2 h postinfection was bound with nearly equal intensity to both phosphorylated forms of CDK2 (Fig. 4B, lane 2). Early postinfection (4 to 6 h), p21WAF1 bound predominantly to the active 33-kDa form of CDK2 in both AAV2-infected and uninfected HFK cultures (Fig. 4B) but, at later times in both AAV2-infected and uninfected HFK cultures, p21WAF1 equally bound to the inactive hyperphosphorylated 34-kDa form of CDK2 (Fig. 4B). Throughout the experiment, total expression of CDK2 in HFK cells also remained unchanged in response to AAV2 infection (Fig. 4D).

In contrast to the observed dynamics of binding between cyclin E and p21WAF1 in AAV2-infected HPV31b-positive cells, the binding pattern between the two proteins was different in HFK cells (compare Fig. 4A and B). At early times, immunoprecipitated p21WAF1 from HFK cultures did not bind cyclin E in AAV2-infected or uninfected samples (Fig. 4B, lanes 1 to 7). Cyclin E-bound p21WAF1 complexes were restored later in infection and could be visualized 10 h postinfection, and they were bound with equal intensity in both uninfected and AAV2-infected cultures (Fig. 4B, lanes 8 to 13). Thus, changes in cyclin E binding to p21WAF1 in HFK cells were unaffected by AAV2 superinfection. Interestingly, restored binding of cyclin E to p21WAF1 at late time points correlated with simultaneous binding exclusively to the hyperphosphorylated 33-kDa (inactive) form of CDK2. The results in Fig. 4 portray cell cycle-dependent p21WAF1-regulated binding of cyclin E/CDK2 complexes in HFK cells, which is in sharp contrast to the pattern observed in CIN-612 9E cells. We have repeated these experiments several times and obtained similar results. The inability of p21WAF1 to bind to cyclin E was not due to the absence of cyclin E in the HFK cells. Rather, cyclin E was expressed in both AAV2-infected and uninfected cells throughout the time points examined (Fig. 4F).

We hypothesize that the differences in cyclin E binding to p21WAF1 observed in the two cell lines are attributable to E7 binding to the C terminus of p21WAF1 in HPV-infected cells, as has been reported elsewhere (25). The HPV18 E7 oncprotein has been shown to associate with cyclin E in complex with both CDK2 and p107 (47). Thus, it is possible that in HPV31b-positive cells, the E7-bound p21WAF1 also interacts with the cyclin E bound to p21WAF1, consequently serving to further stabilize the interaction of cyclin E with p21WAF1. In comparison, in HFK cells, association of cyclin E with p21WAF1 appears to be dynamic in nature (compare Fig. 4A with B). The differences in the observed binding patterns of cyclin E to p21WAF1 provide the scope for a testable hypothesis which can be addressed in the future using in vitro reagents and techniques.

It is to be noted that variabilities in p21WAF1 protein expression observed between different experiments, such as those observed between Fig. 2 and 4, are inherent to the cycling nature of the cells and the nature of the biological system in hand. Thus, some variability in protein expression at the different time points would be expected from cell cycle studies presented here. However, since we consistently compared the effect of AAV2 on p21WAF1 expression to that in uninfected mock samples at each time point tested, it becomes more important to compare trends in changes of protein expression rather than actual amounts of proteins present in the mock control samples at each time point, in each experiment. Thus, the trends in the changes of protein expression are notable, i.e., in HPV-positive cells, AAV2 decreased p21WAF1 expression compared with uninfected controls, whereas in HFK cells AAV2 increased p21WAF1 expression compared with uninfected controls. As p21WAF1 is the central protein of our study, we have graphically represented changes in p21 protein expression together with error bars for this purpose (Fig. 3). The observed variability of changes in p21 expression between experiments was not considerable, as shown by the size of the error bars.

**Modulation of p21WAF1 protein levels is not a consequence of changes in p53 expression.** The expression of p21WAF1 is regulated by both p53-dependent and -independent pathways (41). Induction of p53 directly correlates with G1 arrest in response to DNA damage (18). Upon DNA damage the p53 tumor suppressor acts as a transcriptional activator and induces p21WAF1 expression via p53 response elements (26). We therefore wished to determine whether the AAV2-mediated changes in p21WAF1 expression in the CIN-612 9E and HFK cell lines could be correlated to changes in p53 levels. In each cell line tested, p53 protein levels remained unchanged upon AAV2 infection (Fig. 4G and H). Our results are in agreement with earlier studies which demonstrated that p53 levels in primary fibroblasts were unaffected upon AAV2 infection (31). Therefore, our results suggest that changes in p21WAF1 expression levels occur via cellular pathways independent of changes in expression of p53. We have repeated these experiments multiple times and obtained similar results. It is to be noted that in our experiments the HPV31b-positive cells maintain appreciable amounts of p53 (Fig. 4) in the presence of the E6 oncogene. The ability to maintain p53 protein levels with an increased half-life in the presence of E6 was also reported earlier (35, 39) and is thought to be a result of E2-mediated inhibition of E6 transcription from the HPV early promoter (37, 61), and our results in the current study are reflective of these earlier reports. In fact, transcriptional control of E6 by the E2 protein is thought to regulate cellular levels of p53 at a critical level in order to create a prolonged G2/M cell cycle block, an environment conducive to continued DNA re-replication in the absence of cell cycle progression (23). Additionally, it is also possible that exposure times required to detect p53 expression in our Western blot assays may be longer for CIN-612 9E cells than for HFK cells, and such measurements have not been carried out here.

The AAV2-modulated decrease in p21WAF1 protein levels in HPV-positive cells is in response to accelerated proteosome-mediated degradation. The ubiquitin-proteosome pathway has been demonstrated to be responsible for controlling levels of p21WAF1 via degradation in a cell cycle-specific manner (11). We investigated whether or not the AAV2-targeted decrease of p21WAF1 levels in HPV31b-positive cells was due to increased proteosome-mediated degradation. AAV2-infected and mock-infected CIN-612 9E cells were treated with MG132, an inhibitor of proteosome-mediated proteolysis (54). The abundance of p21WAF1 in response to MG132 treatment was evaluated over a 24-h period and compared in parallel to
elevated levels of p21WAF1 in the uninfected cells (Fig. 5, lanes 18 h and 24 h postinfection, as can be discerned from the way continued to be inhibited in response to MG132 as late as via the proteosome pathway. Activity of the proteosome path-

cells is due to accelerated degradation of this CDK inhibitor histone H1 kinase activity correlates with decreased p21WAF1 (Fig. 6). The AAV2-mediated increase in CDK2-associated and generally remained upregulated following AAV2 infection CIN-612 9E cultures was increased as early as 2 h postinfection 

The AAV2-mediated decrease in p21WAF1 levels correlates with increased CDK2-associated kinase activity. A G1-phase increase in p21WAF1 protein levels functions to inhibit CDK2-associated kinase activity by binding with cyclin E/CDK2 complexes (66). Formation of higher-order complexes with p21WAF1 prevents Rb phosphorylation in late G1, and the start of S phase of the cell cycle (63). On the other hand, p21WAF1 levels are low during S-phase entry and progression (40) and are generally correlated with an increase in CDK2-associated kinase activity (65). We wished to determine whether AAV2-targeted p21WAF1 degradation in the HPV31b-positive cell line affected CDK2-associated kinase activity. We immunoprecipitated CDK2 from CIN-612 9E and HFK mock-infected and AAV2-infected cultures, followed by analysis of CDK2-associated histone H1 kinase activity (Fig. 6). Our results demonstrated that CDK2-associated kinase activity in AAV2-infected CIN-612 9E cultures was increased as early as 2 h postinfection and generally remained upregulated following AAV2 infection (Fig. 6). The AAV2-mediated increase in CDK2-associated histone H1 kinase activity correlates with decreased p21WAF1 protein levels in AAV2-infected HPV31b-positive cells (compare Fig. 6 with Fig. 2). In contrast to the situation in CIN-612 9E cells, AAV2-infected HFK cultures in general displayed decreased CDK2-associated kinase activity compared to uninfected cultures at each time point examined (Fig. 6). The decreased kinase activity correlated with increased p21WAF1 protein levels in AAV2-infected HFK cells (compare Fig. 6 with Fig. 2). Overall, our results from these experiments suggest that AAV2-targeted proteosome-regulated degradation of p21WAF1 in HPV31b-positive cells (Fig. 5) effectively relieves the inhibition of CDK2 kinase activity by the p21WAF1 CDK inhibitor (Fig. 6), resulting in a general increase in CDK2-associated kinase activity.

Increased cyclin E expression following AAV2 infection correlates with increased CDK2-associated kinase activity. To be active, CDK subunits must complex with their respective cyclin components. The increased CDK2-associated H1 kinase activity specific in AAV2-infected CIN-612 9E cells could result from the association either with cyclin E or cyclin A subunits. Cyclin E/CDK2-associated kinase activity is required to mediate Rb phosphorylation in late G1, and catalyze the G1/S transition (64), whereas cyclin A/CDK2-associated kinase activity is required for S-phase progression (56). In order to determine the identity of the cyclin subunit potentially involved in CDK activation, cyclin E and cyclin A expression was studied. In CIN-612 9E cells, cyclin E levels were shown to increase upon AAV2 infection and remained generally elevated (Fig. 7A and 4E). It has been previously reported that in normal cells, cyclin E expression is also transiently upregulated at the G1/S border (65). In contrast to cyclin E, cyclin A levels show decreased expression in AAV2-infected HPV31b-positive cells (Fig. 7A) and were reminiscent of cellular conditions nonpermissive for S-phase entry and progression (65). As would be expected in the mock-infected samples, both cyclin E and cyclin A protein

![Image](http://jvi.asm.org/)

FIG. 5. AAV2 targets p21WAF1 for accelerated proteosome-mediated degradation. CIN-612 9E cells were infected with AAV2 as described in Materials and Methods. A duplicate set of cells infected with AAV2 was treated with MG132 proteosome inhibitor at a final concentration of 5 μM. Cell extracts were prepared and used to immunoprecipitate p21WAF1 as described in Materials and Methods. Immunoprecipitates were resolved on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose, followed by Western blotting as discussed above using polyclonal antibodies against p21WAF1.

![Image](http://jvi.asm.org/)

FIG. 6. CIN-612 9E cells display increased CDK2-associated kinase activity in response to AAV2 infection. CIN-612 9E cells (top panel) and HFK cells (bottom panel) were infected with AAV2 as described in Materials and Methods. Cell extracts were prepared, and immunoprecipitations were performed utilizing a polyclonal antibody against CDK2. Immunoprecipitates were subjected to kinase reactions as described in Materials and Methods, resolved on a 12% polyacrylamide gel, and autoradiographed.
levels fluctuated across the 24-h time period examined. Our results suggest that the early increase in CDK2-associated kinase activity in response to AAV2 infection of HPV31b-positive cells (Fig. 6) is most probably mediated by cyclin E/CDK2 complexes and not cyclin A/CDK2 complexes. In support of this, immunoprecipitation experiments showed that cyclin E but not cyclin A (data not shown). In contrast, in HFK cultures, both cyclin E and cyclin A protein expression was unresponsive to AAV2 infection (Fig. 7B).

Our results demonstrated that AAV2 upregulates cyclin E expression in HPV31b-positive cells (Fig. 7A and 4E). In addition, AAV2 upregulation of CDK2-associated H1 kinase activity (Fig. 6) correlates with this increase in cyclin E expression. Taken together, our results suggest that AAV2 infection results in increased cyclin E/CDK2 complexes which have the potential to enhance pRb phosphorylation. In normal cells, cyclin E/CDK2-mediated phosphorylation of pRb in late G1 enhances the G1/S transition checkpoint (64). However, we were unable to detect changes in pRb phosphorylation in either CIN-612 9E or HFK cultures in response to AAV2 infection (compare Fig. 7A and B). The very low levels of pRb in HPV31b-positive cells compared with HFK cells are due to degradation activities of the E7 oncoprotein on the pRb protein. These results indirectly suggest that, at least within the 24-h time period tested, functional activities of the E7 oncoprotein are not a target for AAV2.

Since E2F is an upstream transcriptional activator for both cyclin E and cyclin A expression, we also determined the expression of the E2F protein. Except for an increase seen at 24 h, E2F expression was generally decreased in AAV2-infected HPV31b-positive cells infected with AAV2 (Fig. 7A). Therefore, our results suggest that AAV2 can differentially regulate expression of these two cyclins, even though transcription of both cyclins is under control of E2F-dependent activity (1).

AAV2 specifically downregulates p21WAF1 protein levels only in HPV-infected keratinocytes. We considered the possibility that the ability of AAV2 to differentially modulate p21WAF1 expression in the CIN-612 9E and HFK cell lines is inherently due to their mucosal and cutaneous sources of origins, respectively. In order to address this possibility, we repeated our experiments using the HPV31a:xb cell line, which was derived by electroporating the HPV31a genome into cutaneous-derived HFK cells (48). The HPV31a:xb line maintains episomal copies of HPV31a and in our hands is capable of producing infectious HPV virions in differentiation-dependent raft cultures (48). Thus, the HPV31a:xb line provides a comparable line to CIN-612 9E with respect to HPV genome maintenance and ability to produce infectious virus, but with the added advantage of having the same genetic background as HFK cells. AAV2-infected HPV31a:xb lines also displayed decreased levels of p21WAF1 expression compared to mock controls at each time point tested (Fig. 8A), similar to that seen with the CIN-612 9E line. CDK2 kinase activity was also upregulated in response to AAV2 infection compared with mock controls (Fig. 8B), similar to that seen with the CIN-612 9E cell line. In contrast, total cellular levels of CDK2 remained unchanged. Thus, these results greatly provide strength for the idea that AAV2 regulation of p21WAF1 protein expression is dependent on the presence of HPV and its gene products.

AAV2 modulation of protein profiles does not correlate with significant changes in cell cycle progression. Thus far, we have established that AAV2 decreased p21WAF1, cyclin A, and E2F levels (Fig. 2A and 7A) and also increased CDK2-associated kinase activity and cyclin E levels in the CIN-612 9E cells (Fig. 2E and 6). We therefore expected that these changes would correlate with increased movement of cells across the G1/S border of the cell cycle, whereas AAV2-infected HFK cultures
would accumulate in the G1 phase. To test this possibility, we performed FACS analysis to determine whether the observed changes in the cell cycle protein profiles correlated with changes in cell cycle progression (Fig. 9). Surprisingly, we were unable to detect any significant differences in cell cycle progression in AAV2-infected cells (Fig. 9) which would correlate with the molecular changes detected in HPV31b-positive and HFK primary cell lines. We have repeated these experiments several times with similar results. It is to be noted that in the present study, the FACS profile obtained for the keratinocyte lines was characteristic of this cell type, as previously reported (43). Therefore, AAV2 infection results in changes at the level of protein expression and activity, but these changes do not appear to perturb the normal steady flow through the cell cycle.

Another possibility is that our observed changes relate to the process of differentiation, which is also closely coupled to changes in cell cycle progression and occurs in the G1 phase of the cell cycle (79). In addition, AAV2 has been shown to upregulate markers of differentiation in naturally occurring tumor-derived lines (2). Thus, it might be predicted that the AAV2-stimulated G1/S-like cellular conditions would correlate with increased cellular differentiation accompanied by increased expression of involucrin, K10, and K14 protein levels in AAV2-infected CIN-612 9E cells. However, we did not observe any changes in expression of these differentiation markers in either cell line tested (data not shown).

**DISCUSSION**

In the current report, we examined the effect of AAV2 infection on cell cycle targets in HPV-infected cells. The highlight of the present study was the observation that AAV2 differentially regulates p21WAF1 protein expression in an HPV31b-positive cervical carcinoma line. Expression of p21WAF1 CDK inhibitor was downregulated in a proteosome-dependent manner in HPV31b-positive cells in response to AAV2, whereas in AAV2-infected HFK primary cells p21WAF1 levels were increased. These results suggest that the presence of HPV and its associated gene products determines the regulation of p21WAF1 protein by AAV2. Our results presented in this communication add to the growing list of cellular proteins targeted by AAV2 during infection of both primary cell lines as well as cell lines infected with oncogenic viruses. Our results suggest that the negative association of AAV2 with cervical cancer may be an end point of cellular events initiated at the early stages of AAV2 infection of HPV-positive cells via targeting of key proteins which control cell cycle progression.
Downstream consequences of lowering p21WAF1. Downregulation of p21WAF1 protein levels in AAV2-infected HPV31b-positive cells had several consequences. Lowering of p21WAF1 levels upon AAV2 superinfection was associated with increased CDK2-associated kinase activity, as would be expected (65). At the same time, cyclin E protein expression was upregulated in HPV31b-positive cells, but cyclin A was not, suggesting that cyclin E/CDK2 complexes were most probably activated as a consequence of lowering p21WAF1 levels. The resultant increase in kinase activity of cyclin E/CDK2 complexes has the potential to phosphorylate Rb in late G1. These results suggest that AAV2 modulates a cellular environment resembling that of an enhanced G1/S junction of the cell cycle in HPV31b-positive cells. In contrast, in HPV31b-positive cells

FIG. 9. Infection of HPV-positive (A) as well as HFK (B) cells with AAV2 does not result in significant changes in cell cycle progression. Both HPV and HFK cells were infected with AAV2 and subjected to FACS analysis as described in Materials and Methods. The percentages of cells in the various cell cycle phases were plotted at the various time points indicated. (C) Complete FACS analysis profiles of AAV2-infected and mock-infected CIN-612 9E cells. (D) Complete FACS analysis profiles of AAV2-infected and mock-infected HFK cells.
E2F protein levels were decreased in response to AAV2 infection. In AAV2-infected HFK cells, increased p21WAF1 protein levels were associated with decreased CDK2-associated kinase activity, as would be predicted, consistent with the role of AAV2 as a tumor-suppressive parvovirus.

The AAV2-mediated upregulation of CDK2-associated kinase activity in HPV-infected cells is interesting given the mechanism of its activation under normal conditions. Generally, in addition to decreased p21WAF1 levels, the phosphatase mechanism of its activation under normal conditions. An enhanced G1/S-like environment could potentially bifurcate the role of E2F transactivation of cell cycle gene expression, at least with respect to cyclin E and cyclin A, or perhaps acts through an E2F-independent pathway.

p21WAF1 is a cell cycle target of AAV2. Our results presented in the current study suggest that p21WAF1 is an early target for AAV2 upon infection of both HPV-infected and also primary keratinocytes. However, downstream consequences of affecting p21WAF1 expression in each cell type appear to be very different. In the current study, in HPV-positive cells, AAV2-induced proteosome-mediated degradation of p21WAF1 in effect lowered inhibition of kinase activity of cyclin E/CDK2 complexes, thus presenting conditions which in normal cells are favorable for crossing the G1/S junction and S-phase progression (40). This is an interesting observation, in light of the fact that AAV2 has been hypothesized to be an antioncogenic virus. Thus, at first glance, the AAV2-targeted decrease in p21WAF1 levels in HPV-infected cells appears to contradict its role as a tumor-suppressive virus and its negative association with cervical cancer. On the other hand, AAV2-mediated p21WAF1 degradation in HPV31b-positive cells is a significant observation, given that p21WAF1 is the universal CDK inhibitor and a pivotal cell cycle regulator (65, 66). The HPV E7 oncoprotein has been shown to deregulate p21WAF1 expression and activity in keratinocytes (25, 61). Since AAV2 has also been shown to counteract deregulated cell cycle targets in adenovirus-infected cells (5–7), our results support the hypothesis that p21WAF1-containing complexes may be a direct or indirect target of AAV2 in HPV-positive cells. The AAV2 targeted decrease of p21WAF1 protein levels was also found to be irrespective of its cellular environment, i.e., whether mucosal or cutaneous in origin, so long as HPV was present in these cells. Our results in this report provide a foundation to dissect the mechanism of AAV2-mediated targeting of p21WAF1-containing complexes and subsequent triggering of downstream cell cycle events specific to the presence of HPV. How these AAV2-generated cell cycle effects may translate into downstream suppression of HPV-related functions remains to be further elucidated in the future by utilizing both in vitro reagents as well as in vivo techniques.

Studies reported elsewhere suggest that AAV gene expression and subsequent replication, similar to that of the autonomous paroviruses, may be strongly dependent on the biochemical milieu of the S phase of the cell cycle (80). Thus, p21WAF1 regulation by AAV2 may only be a small, but important, mobilization step in the cascade of events which may be favorable for AAV2-specific functions, which may lead to AAV2’s counteraction of HPV-specific functions.

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REFERENCES


