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Functional Consequences of Directed Mutations in Human Papillomavirus E6 Proteins: Abrogation of p53-Mediated Cell Cycle Arrest Correlates with p53 Binding and Degradation *in Vitro*

ROBBERT J. C. SLEBOS,[1] THEODORE D. KESSIS, ALEX W. CHEN, SUNG M. HAN, LORA HEDRICK,[2] and KATHLEEN R. CHO[2]

Department of Pathology, The Johns Hopkins University School of Medicine, 656 Ross Research Building, 720 Rutland Avenue, Baltimore, Maryland 21205-2196

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Clinical and epidemiological studies have implicated the involvement of human papillomavirus (HPV) infection in cervical tumorigenesis. We have previously shown that expression of high-risk (HPV16) E6 can abrogate an important cell cycle checkpoint mediated by p53. Sublethal DNA damage causes p53 accumulation and G_1 arrest in normal cells, but not in cells with mutant or absent p53, or in cells that express HPV16-E6. To investigate the functional consequences of low-risk (HPV11) E6 expression and to evaluate regions of E6 believed to mediate interaction with p53, we generated several E6 expression constructs, including HPV11-E6, and four different E6 mutants. HPV16E6ΔD and HPV16E6ΔB had short deletions of nucleotides encoding amino acids previously implicated in p53 degradation and binding, respectively. HPV16E6HL and HPV11E6LH had the putative p53 binding domain exchanged between the high- and the low-risk types. Unlike HPV16-E6, HPV11-E6 and the mutant E6 proteins were not able to bind or degrade p53 in *in vitro* assays. When expressed in RKO cells, HPV11-E6 or the mutant E6 proteins did not prevent p53 accumulation or interfere with p53-dependent *WAF1/CIP1* mRNA expression, allowing p53-mediated G_1 cell cycle arrest after DNA damage. These findings demonstrate that low-risk and high-risk E6 proteins differ in their effects on p53-mediated cell cycle control and that rather subtle mutations of high-risk E6 can alter its ability to abrogate this important cellular response.

INTRODUCTION

Numerous clinical and epidemiological studies have found a strong correlation between cervical cancer and infection with certain types of human papillomavirus (HPV). This association represents one of the few instances where viral infection is implicated in human tumorigenesis and therefore provides a valuable model system in which to study the cellular targets of viral proteins. Approximately one-third of the more than 70 known HPV types are capable of infecting the lower genital tract. Of these, the high-risk types (such as 16 and 18) are associated with high-grade squamous intraepithelial lesions and invasive cervical carcinomas, whereas the low-risk types (such as 6 and 11) are more often found in low-grade intraepithelial lesions (zur Hausen, 1989).

Biological studies have implicated two of the early HPV genes in the neoplastic process, E6 and E7. In combination, these genes are capable of immortalizing primary human keratinocytes (Barbosa and Schlegel, 1989; Hawley-Nelson *et al.*, 1989). The E7 oncoprotein binds the product of the retinoblastoma tumor suppressor gene (pRB) and may serve to inactivate pRB in the cell (Dyson *et al.*, 1989). The E6 protein of the high-risk HPVs can bind the p53 tumor suppressor protein *in vitro* (Werness *et al.*, 1990) and enhance the degradation of p53 via a ubiquitin-mediated pathway (Scheffner *et al.*, 1990). Low-risk HPV E6 does not promote the degradation of p53 in these *in vitro* assays (Crook *et al.*, 1991; Scheffner *et al.*, 1990). A third protein, E6-AP, mediates the interaction between E6 and p53 (Huibregtse *et al.*, 1991), and there is evidence for a direct role of E6-AP in p53 ubiquitination (Scheffner *et al.*, 1993). Crook *et al.* have localized putative HPV16-E6 sequences required for p53 binding and enhancement of p53 degradation (Crook *et al.*, 1991). A carboxyl-terminal region conserved among all HPV types was found to be important for p53 binding, while an amino-terminal domain did not mediate p53 binding, but appeared to be necessary for p53 degradation. Studies using chimeric low-risk/high-risk E6 proteins provide strong evidence of distinct N-terminal domain(s) mediating p53 degradation (Crook *et al.*, 1991; Foster *et al.*, 1994). However, the existence of distinct domains mediating p53 binding remains a controversial issue as some

[1] Current address: Department of Pathology, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1104 AZ Amsterdam, The Netherlands.

[2] To whom correspondence and reprint requests should be addressed: Fax: (410) 614-3548.

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investigators have been unable to demonstrate binding of E6 proteins encoded by low-risk HPVs to p53 (Werness *et al.*, 1990).

Although the combination of high-risk E6 and E7 can immortalize primary human cells, a fully transformed phenotype is rarely achieved and usually appears only after numerous passages. In addition, only a small minority of HPV-infected women eventually develop invasive cervical carcinomas. These observations suggest that additional genetic alterations are needed in HPV-infected cells for the development of a malignant lesion. One mechanism by which such genetic alterations may arise is through replication of damaged DNA.

Normal cells respond to sublethal DNA damage with cell cycle arrest in G_1 (Kuerbitz *et al.*, 1992). One of the regulatory signals in the G_1 arrest pathway appears to be accumulation of the p53 tumor suppressor protein (Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992). Cells with impaired p53 function (due to absent or mutated p53 genes) fail to accumulate p53 after DNA damage and do not arrest in G_1 (Kastan *et al.*, 1992). Impaired p53 function has been associated with certain types of genetic instability, such as gene amplification and aneuploidy (Livingstone *et al.*, 1992; Yin *et al.*, 1992; Carder *et al.*, 1993). Human fibroblasts in which p53 function has been inactivated by expression of high-risk HPV E6 protein show aneuploidy and acquisition of the ability to amplify drug resistance genes (White *et al.*, 1994). Thus, failure of cells to undergo p53-dependent G_1 arrest after DNA damage may lead to the fixation of mutations in daughter cells, resulting in the accumulation of genetic alterations required for tumor development and progression.

RKO colorectal carcinoma cells express wild-type p53 protein and demonstrate a normal DNA damage response. We have previously reported that high-risk (HPV16) E6 expression in RKO cells disrupts this response (Kessis *et al.*, 1993). Furthermore, these cells showed markedly reduced levels of p53 protein before and after DNA damage, most likely reflecting E6-mediated degradation of p53 *in vivo*. To investigate further the functional effects of E6 on the p53-mediated cell cycle checkpoint, we expressed low-risk (HPV11) E6 in RKO cells. In addition, we generated several expression constructs with E6 mutants containing deletions or alterations in regions of E6 that have previously been implicated in p53 binding and/or degradation. All E6 proteins were tested in *in vitro* assays for p53 degradation and binding and were expressed in RKO cells to test their effects on DNA damage-induced p53 accumulation, p53-dependent induction of *WAF1/CIP1* mRNA expression, and p53-mediated cell cycle control. HPV16-E6 was able to bind and promote the degradation of p53 *in vitro* and to disrupt the normal response to DNA damage in RKO cells. Significant p53-dependent induction of *WAF1/CIP1* expression was not observed in HPV16-E6-expressing cells. In contrast, we could not demonstrate binding or degradation of p53 by HPV11-E6 *in vitro*. Expression of HPV11-E6 in RKO cells did not interfere with p53 accumulation, *WAF1/CIP1* mRNA induction, or the G_1 arrest after DNA damage. None of the mutant E6 proteins were capable of binding or degrading p53 *in vitro*, and all behaved essentially as low-risk E6 in the *in vivo* functional assays.

MATERIALS AND METHODS

Plasmid constructs and mutagenesis

The open reading frame of HPV16-E6 (map positions 42–527) was amplified by PCR and cloned in the unique *Bam*HI cloning site of the eukaryotic expression vector pCMVneo (Baker *et al.*, 1990) as described previously (Kessis *et al.*, 1993). This HPV16-E6 fragment was also subcloned in the plasmid pAlter to introduce desired mutations using a site-directed mutagenesis kit (Promega). Putative E6 domains believed to mediate p53 binding and degradation *in vitro* have been mapped (Crook *et al.*, 1991). Amino acids 106–115 (nucleotides 419–449) were found to be important for p53 binding, while amino acids 9–13 (nucleotides 128–142) and 45–49 (nucleotides 236–250) were found to be important for mediation of p53 degradation. For this study, three different mutants of HPV16-E6 were constructed: (1) 16E6ΔD, lacking amino acids 8–12 (nucleotides 125–139); (2) 16E6ΔB, lacking nucleotides 422–439 (amino acids 107–112); and (3) 16E6HL, with substitutions of nucleotides 422–439 in the putative p53 binding domain to those found in low-risk HPV types. The (antisense) oligonucleotides used to generate these constructs were: (1) 5′-GTA CAT AAC TGT GGC TCC TGT GGG TC-3′ (nt 113–153), (2) 5′-GTC TTT GCT TTT CCT CAC AGT TAA TAC ACC-3′ (nt 408–455), and (3) 5′-GCT TTT CTA CCT CAC TCA GTT TCG AGT GAC AGT T-3′ (nt 416–449). A schematic representation of the vector pCMVneo and the different HPV16-E6 constructs is given in Fig. 1.

In addition, an expression construct containing HPV11-E6 was made starting from the plasmid PJ6Ω-11E6 (provided by Dr. Atilla Lorincz). The open reading frame of HPV11-E6 (map positions 88–575) was cloned in the unique *Bam*HI site of pCMVneo. This HPV11-E6 *Bam*HI fragment was also subcloned in pAlter (Promega) and mutagenized between nucleotides 417 and 450 (amino acids 108–113) to generate the construct 11E6LH. Amino acids 108–113 were altered to encode the amino acid sequence found in the putative p53 binding domain of HPV16 (see Fig. 1). The (antisense) oligonucleotide used to mutagenize HPV-11E6 was: 5′-GTT TTT CTT CTG GAC ACA ACG GCT TCT GAC ACA G-3′ (nt 417–450). All mutations were confirmed by bidirectional sequence analysis of the entire E6 insert using the Sequenase Version 2.0 sequencing kit (USB).

Protein translation, HPV E6/p53 binding, and p53 degradation *in vitro*

Each of the HPV E6 DNA inserts were subcloned from pAlter into pGEM7 (Promega) for *in vitro* protein translation. A pBluescript construct with the complete coding region of wild-type p53 (provided by Dr. Bert Vogelstein) was used for *in vitro* translation of p53. Proteins were translated in the T_NT coupled transcription/translation system (Promega) using [^{35}S]cysteine as a radiolabel. A typical reaction contained 100 ng of plasmid DNA in a total volume of 50 μl.

GST–p53 fusion proteins were purified from 500-ml cultures of DH5α bacterial cells and coupled to GST–Sepharose beads (Pharmacia) as described previously (Huibregtse *et al.*, 1991). Ten microliters of E6 protein translate was incubated with the p53–GST–Sepharose beads in NP-40 buffer (0.1 *M* Tris, pH 7.4, 0.1 *M* NaCl, 1% NP-40) supplemented with 36 m*M* NaCl (final concentration) for 4 hr on ice. Beads were washed three times with NP-40 buffer, boiled in Laemmli sample buffer, and size separated by SDS–PAGE (12% polyacrylamide).

Degradation of p53 in the presence of E6 was determined essentially as previously described (Scheffner *et al.*, 1990). Briefly, 5 μl E6 translate, 1 μl p53 translate, and 4 μl fresh rabbit reticulocyte lysate (Promega) were incubated in 25 m*M* Tris, pH 7.5, 0.1 *M* NaCl, 3 m*M* DTT for 4 hr at 25° in a total volume of 40 μl. After addition of an equal volume of 2× Laemmli sample buffer, the proteins were analyzed by SDS–PAGE (12% polyacrylamide).

Cell lines and transfections

Logarithmically growing RKO cells were transfected with the pCMVneo/E6 plasmid constructs using Lipofectin as described by the manufacturer (Gibco-BRL). Transfected cells were initially selected in medium containing 1.0 mg/ml G418 (Gibco-BRL) and, after 10 days, single colonies were cloned by limiting dilution in medium with 0.5 mg/ml G418. The HPV16-E6-expressing lines (RC10-1, RC10-2, and RC10-3), and the control RKO cell line transfected with pCMVneo vector alone (RCneo-1), have been described previously (Kessis *et al.*, 1993). The other pCMVneo expression constructs were used to generate multiple clonal lines: 16E6ΔB (RC61-2, RC61-4), 16E6ΔD (RC73-5, RC73L-4), 16E6HL (RC33-2, RC33-9), 11E6LH (RC41-5, RC41-9), and 11E6 (RC11-6, RC11L-2). Each line was independently derived as determined by DNA blotting, and the identity of the transfected DNAs was confirmed by amplifying the full-length E6 from genomic DNA by PCR. These PCR products were cloned and sequenced *en masse* as described previously (Nigro *et al.*, 1989).

E6 and *WAF1/CIP1* mRNA expression in RKO cell lines

For the detection of mRNA expression, cells were grown to 80% confluency and total cellular RNA was isolated from unirradiated cells or from cells 4 hr after irradiation with 4 Gy, as previously described (Chomczynski and Sacchi, 1987). Total RNA (20 μg) was size fractionated on 1.5% agarose MOPS/formaldehyde gels, transferred to nylon membranes, and hybridized to ^{32}P-labeled HPV E6 or *WAF1/CIP1* (*WAF1/CIP1* cDNA was kindly provided by Dr. B. Vogelstein) probes in rapid hybridization buffer (Amersham). The blots were washed at 65° in 0.3× SSC/0.1% SDS and then autoradiographed. The same blots were hybridized either separately (for E6) or simultaneously (for *WAF1/CIP1*) to a GAPDH (glyceraldehyde phosphate dehydrogenase) cDNA probe as a control for possible variation in sample loading.

p53 protein detection

The monoclonal antibody DO-1 (Oncogene Science) was used to detect steady-state levels of p53 protein on immunoblots. Accumulation of p53 was measured at 4 hr after 4 Gy of γ-irradiation in a ^{137}Cs irradiator and compared to cells that received no treatment. For this analysis, 2×10^6 cells were lysed in Laemmli sample buffer and quantitated on dot blots with amido black using protein standards. Approximately 100 μg of protein was size separated by SDS–PAGE, electroblotted onto nitrocellulose membranes, and probed with p53 antibodies. Enhanced chemiluminescence (ECL; Amersham) was used for final detection of the immunoreactive protein.

Cell cycle analysis

RKO cell lines were analyzed for cell cycle distribution as previously described (Kuerbitz *et al.*, 1992; Kastan *et al.*, 1992; Kessis *et al.*, 1993). Briefly, cells were grown to 80% confluency, and treated with 0 or 4 Gy of γ-irradiation. Seventeen hours post-treatment cells were labeled for 4 hr with 10 μM 5-bromodeoxyuridine and then stained for DNA synthesis using fluorescein isothiocyanate-conjugated anti-5-bromodeoxyuridine antibody (Becton–Dickinson). Cells were also stained for total DNA content with propidium iodide. The 17-hr time point and 4 Gy of γ-irradiation have previously been shown to accurately reflect p53-mediated G_1 arrest in the RKO cell line (Kastan *et al.*, 1991, 1992). Each individual cell line was assayed in at least three independent experiments (10,000 cells/experiment).

RESULTS

Generation of E6 mutants

A systematic study by Crook *et al.* provided data suggesting that distinct regions of HPV E6 are important for

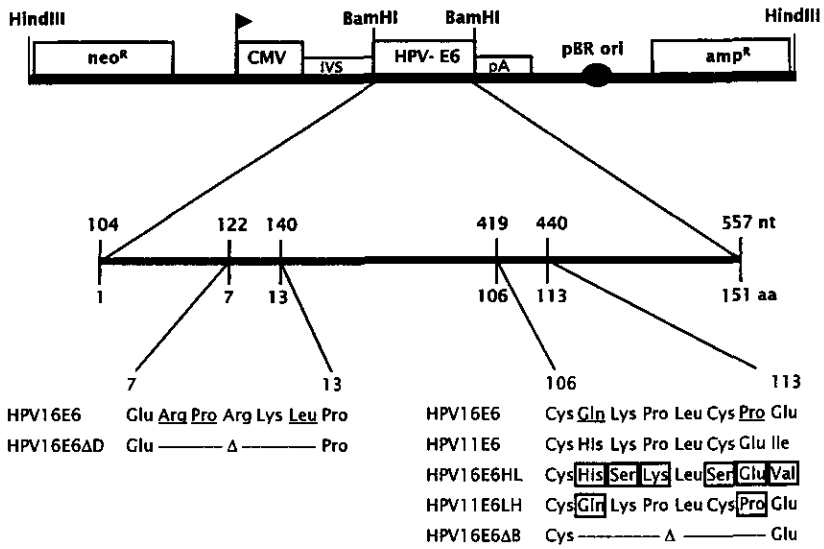

FIG. 1. Schematic representation of the eukaryotic expression vector pCMVneo with various HPV E6 inserts. In pCMVneo, constitutive expression of inserted sequences is driven by a CMV promoter. All inserts were cloned downstream of rabbit β-globin intervening sequence (IVS) and upstream of the rabbit β-globin polyadenylation signal (pA). Two regions of HPV E6 were mutated: amino acids 8–12 and amino acids 107–112. The amino acids conserved among the oncogenic HPVs are underlined, and the positions that were altered are in boxes. In addition to wild-type HPV16 and 11, four mutants were generated: HPV16E6ΔD, deleting a region previously reported to be involved in p53 degradation; HPV16E6ΔB, deleting a putative p53 binding domain; HPV16E6HL, with an exchange of amino acids conserved among high-risk HPVs to those of low-risk types in the putative binding domain; and HPV11E6LH, with an exchange of amino acids from low-risk HPVs to those of high-risk HPVs in this region.

the binding and degradation of p53 *in vitro* (Crook *et al.*, 1991). Deletion of amino acids 106–110 and 111–115 virtually abolished the ability of HPV16-E6 to bind and degrade p53, while other deletions in HPV16-E6 appeared to affect the association between E6 and p53 to a lesser extent or not at all. E6 from low-risk type HPVs was found to bind to p53 (Crook *et al.*, 1991), albeit with substantially lower affinity than E6 from high-risk HPV types. Other investigators, however, have been unable to demonstrate binding of low-risk E6 to p53 (Werness *et al.*, 1990).

The region between positions 106 and 115 in HPV16-E6 contains several amino acids that are conserved between all HPV types (positions 106-Cys, 110-Leu, 114-Glu, and 115-Lys) and two positions that are conserved among oncogenic HPVs (positions 107-Gln and 112-Pro). To study alterations of this putative p53 binding domain, we generated three different E6 mutants (Fig. 1). The first, HPV16E6ΔB, contains a deletion of amino acids 107–112 (including the two positions conserved among oncogenic HPVs). In the second construct, HPV11E6LH ("Low-to-High"), the low-risk HPV11-E6 sequence between positions 107 and 112 was replaced with the corresponding high-risk HPV16 sequence. Since single point mutations in this region did not alter the properties of high-risk E6 (Crook *et al.*, 1991), we chose to generate the third construct HPV16E6HL ("High to Low") such that six amino acids were exchanged with those frequently found in low-risk viruses or in HPVs not associated with genital tract lesions.

The N-terminal portion of HPV16-E6 contains at least two putative domains important for mediating p53 degradation. Codons 8–12 encode three amino acids conserved among high-risk HPV types (positions 8-Arg, 9-Pro, and 12-Leu). Crook *et al.* found that deletion of amino acids 9–13 markedly reduced E6-mediated degradation of p53 but had no effect on p53 binding (Crook *et al.*, 1991). In addition, amino acid substitutions of HPV16-E6 at positions 45, 47, and 49 to those conserved in low-risk HPV types similarly affected p53 degradation but not p53 binding. Point mutations altering only one of the three conserved amino acids in this region failed to substantially affect p53 binding or degradation. We chose to evaluate further the most N-terminal domain mediating p53 degradation. A fourth construct (HPV16E6ΔD) was generated by deleting amino acids 8–12 from HPV16E6 (Fig. 1). This deletion eliminates all three amino acids conserved among high-risk HPVs in this region.

All wild-type and mutant E6 DNA inserts were subcloned into pCMVneo (Baker *et al.*, 1990) and pGEM7 (Promega) for eukaryotic expression and coupled *in vitro* transcription/translation, respectively.

E6/p53 binding *in vitro*

Two p53–glutathione *S*-transferase plasmid constructs (kindly provided by Dr. J. Huibregtse) were used to determine *in vitro* p53-binding capacity of the different E6 proteins. The first construct, p53–GST, encodes a wild-type p53–GST fusion protein which efficiently binds

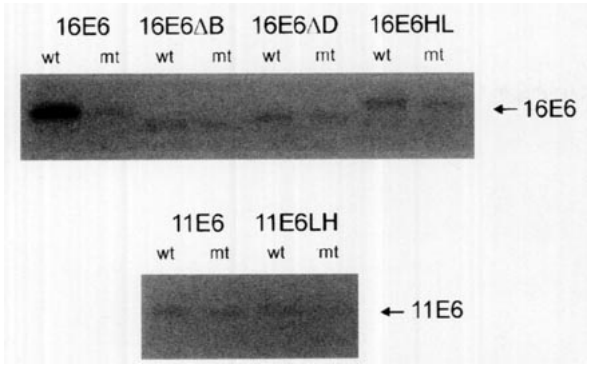

FIG. 2. Association of E6 with p53 *in vitro*. GST–p53 fusion proteins were purified from bacterial cultures, coupled to GST–Sepharose beads, incubated with labeled HPV E6 translated *in vitro*, and bound material was fractionated by SDS–PAGE and visualized by autoradiography. GST fusion proteins contained either the wild-type p53 sequence (wt) or a mutant p53 harboring a tyrosine at position 135 (mt) as a negative control. Specific binding was observed only between wild-type HPV16-E6 and the wild-type p53–GST fusion protein.

high-risk HPV E6, while the second, $p53^{135Tyr}$–GST, has a missense mutation at codon 135 (Cys to Tyr). This mutant p53–GST fusion protein does not bind E6 (Huibregtse *et al.*, 1991). The results of a representative experiment are shown in Fig. 2. HPV16-E6 bound the wtp53–GST fusion, while HPV11-E6 and the various E6 mutants, including HPV16E6ΔD, all failed to bind wtp53 at detectably greater levels than to the mtp53–GST fusion control. All of the E6 proteins showed low levels of nonspecific interaction with both fusion proteins. Incubation of HPV16-E6 and HPV11-E6 with GST beads alone resulted in no detectable binding (data not shown).

E6-mediated p53 degradation *in vitro*

High-risk HPV16-E6 has been shown to enhance the degradation of p53 *in vitro* via a ubiquitin-mediated pathway (Scheffner *et al.*, 1990, 1993). This *in vitro* degradation assay was used to determine the functional activity of the E6 proteins (Fig. 3). Incubation of radiolabeled p53 with wild-type HPV16-E6 at 25° resulted in virtual absence of detectable p53 after 4 hr. Incubation of p53 with HPV16-E6 at 4° had no effect on p53 levels (results not shown). Degradation of p53 was not observed following incubation with low-risk HPV11-E6 or with any of the mutant E6 proteins.

Expression of E6 in RKO transfectants

To study the functional consequences of E6 expression, we transfected the low-risk (HPV11) and high-risk (HPV16) E6 and the four mutant E6 constructs into RKO colorectal carcinoma cells. We generated at least two independent cell lines for each E6 construct and tested them for E6 mRNA expression before and 4 hr after 4 Gy of γ-irradiation. E6 expression in representative lines is shown in Fig. 4 (top). The levels of E6 expression did not vary substantially between the different clones, except for the cell lines RC73-5 (expressing 16E6ΔD) and, especially, RC11-6 (expressing HPV11-E6), which showed higher levels of E6 expression relative to GAPDH. DNA blots revealed integration of multiple tandemly repeated copies of HPV11-E6 in RC11-6, accounting for the high E6 expression in these cells (results not shown). RC73-5 showed no apparent increase in HPV E6 DNA copy number. E6 mRNA expression was unaltered by γ-irradiation in all cell lines.

p53 protein levels after DNA damage

In normal cells, p53 protein accumulates transiently in response to DNA damage and is associated with arrest in the G_1 phase of the cell cycle (Kastan *et al.*, 1992). Accumulation of p53 protein was readily detected in parental RKO cells and in RKO cells expressing HPV11-E6 (Fig. 5). In contrast, p53 protein in the HPV16-E6-transfected cell line RC10-1 was only detectable after prolonged (60 min) exposure of the blots. In RC10-1, p53 levels still increased after DNA damage, indicating that the mechanism responsible for p53 protein accumulation in this response remains intact. However, the markedly reduced p53 protein level following γ-irradiation of HPV16-E6-transfected RKO cells is apparently inadequate to mediate cell cycle arrest. In cells transfected with HPV11-E6, and in the cell lines transfected with the four E6 mutants, the p53 protein levels before and after γ-irradiation were similar to those in the control RKO cell lines (Fig. 5). This finding is consistent with the results

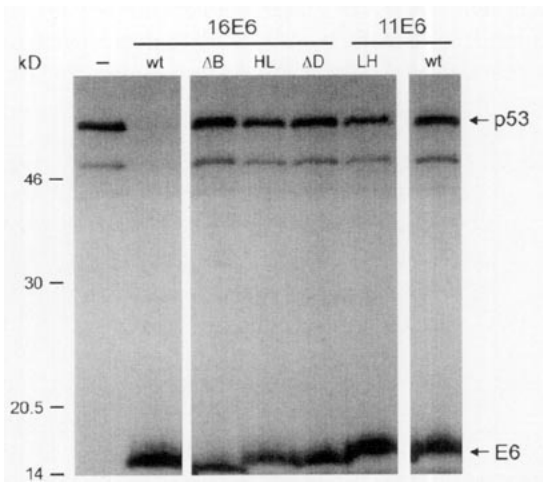

FIG. 3. E6-mediated degradation of p53 *in vitro*. Radiolabeled HPV E6 and p53 proteins were translated *in vitro*, incubated for 4 hr at 25°, and then analyzed by SDS–PAGE. The major p53 and E6 protein species are indicated. *In vitro*-translated p53 alone is shown in lane 1. The remaining lanes show results following incubation of each E6 protein with p53. Degradation of p53 is only apparent following incubation with HPV16-E6.

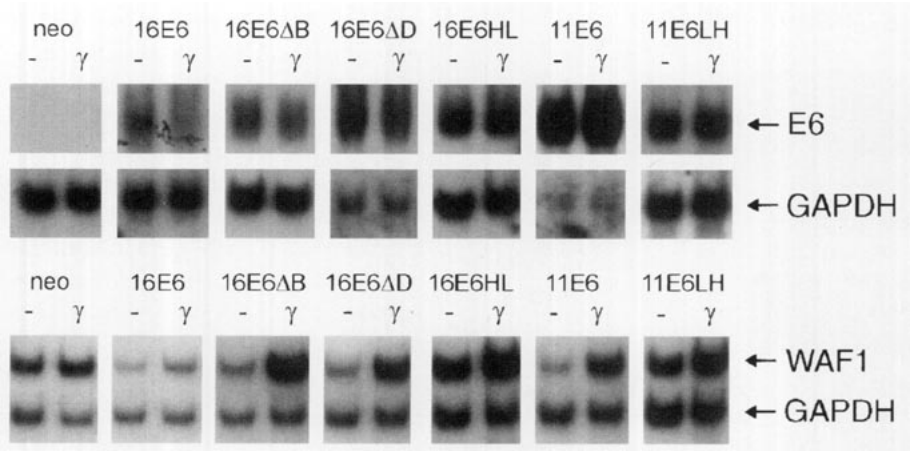

FIG. 4. RNA blot analysis of HPV E6, *WAF1/CIP1,* and GAPDH expression in representative cell lines with and without γ-irradiation. (Top) Expression of E6 was detected in all lines, except in RKO cells transfected with vector alone (RCneo-1). E6 mRNA expression did not vary following γ-irradiation. (Bottom) Expression of *WAF1/CIP1* was induced after γ-irradiation in the cell lines expressing vector alone (neo), 11E6, 16E6ΔB, 16E6ΔD, 16E6HL, and 11E6LH. No significant induction was observed with the 16E6-transfected lines (16E6).

of E6-mediated degradation of p53 *in vitro* using constructs with the identical set of E6 genes.

p53-dependent induction of *WAF1/CIP1* expression

Wild-type p53 has been shown to activate expression of several cellular genes, including *GADD45* (Kastan *et al.,* 1992), *MDM2* (Barak *et al.,* 1993), and a recently described gene variably called *WAF1* (El-Deiry *et al.,* 1993), *CIP1* (Harper *et al.,* 1993), or *SDI1* (Noda *et al.,* 1994). This gene encodes a 21-kDa protein (Xiong *et al.,* 1993) that functions as a general inhibitor of cyclin-dependent kinases. We have recently shown that the p53-dependent cellular response to DNA damage is associated with induction of *WAF1/CIP1* expression (Slebos *et al.,* 1994), presumably one of the signals leading to cell cycle arrest. To investigate the effects on p53-mediated transactivation of *WAF1/CIP1* expression in the HPV E6-transfected RKO cell lines, we analyzed *WAF1/CIP1* mRNA levels before and after sublethal DNA damage (Fig. 4, bottom). After 4 Gy of γ-irradiation, *WAF1/CIP1* expression is induced in the vector-only transfectant (neo) and the low-risk (HPV11E6) cell lines. Two independent RKO cell lines expressing high-risk (HPV16) E6 showed only slight (Fig. 4, bottom, lanes 3 and 4) or no (data not shown) p53-mediated transactivation of *WAF1/CIP1.* The cell lines containing the mutant forms of E6, HPV16E6ΔD, HPV16E6ΔB, HPV16E6HL, and HPV11E6LH, showed a two- to sevenfold increase in *WAF1/CIP1* mRNA levels after γ-irradiation, correlating with the accumulation of functional wild-type p53 in these lines. The induced levels of *WAF1/CIP1* expression in all of these clones are substantially higher than that observed in RKO cells expressing high-risk E6, supporting the notion (Zhang *et*

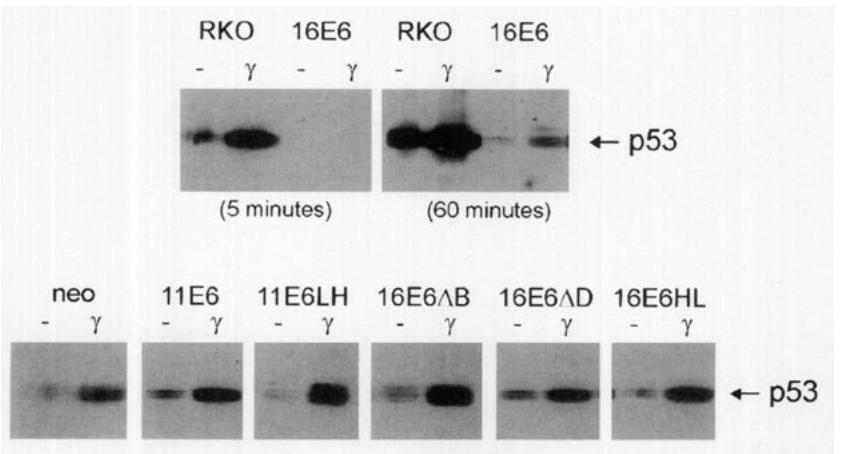

FIG. 5. Immunoblot analysis of p53 before and after 4 Gy of γ-irradiation in representative cell lines. (Top) p53 protein levels in parental RKO cells and in HPV16-E6-expressing RKO cells (RC10-1). The markedly reduced levels of p53 are detectable in HPV16-E6-transfected cells only after prolonged exposure (60 min). (Bottom) p53 levels in RKO cells transfected with vector alone (neo) and expressing HPV11-E6 and E6 mutants (5-min exposure). p53 accumulation remains intact in these cells and levels before and after irradiation are comparable to those of control cells.

al., 1994) that a threshold level of $p21^{waf1/cip1}$ expression is required to bring about DNA damage-induced growth arrest.

Cell cycle control in E6-transfected RKO cell lines

The p53-mediated DNA damage response can be elicited by sublethal doses of DNA strand-breaking agents such as γ-irradiation (Nelson and Kastan, 1993; Fritsche *et al.*, 1993). To study the functional consequences of HPV E6–p53 interactions, we determined cell cycle distribution before and after sublethal DNA damage induced by γ-irradiation (Fig. 6A). In parental RKO cells, and in pCMVneo- and HPV11-E6-transfected lines, irradiation with 4 Gy resulted in G_1 and G_2 cell cycle arrest. When HPV16-E6 was expressed in RKO cells the G_1 arrest was completely lost, although the p53-independent G_2 arrest remained intact (Fig. 6A). Cell cycle analysis of RKO control cells and RKO cells expressing HPV16-E6, HPV11-E6, and the mutant forms of E6 are summarized in Fig. 6B. Cell lines expressing HPV11-E6 and mutant forms of E6 showed a DNA damage response similar to that of untransfected RKO cells, although the levels of cell cycle arrest varied somewhat between individual clones.

DISCUSSION

The molecular basis for variation in oncogenic potential between the high-risk and low-risk types of HPVs remains unclear but is of considerable interest with respect to understanding the molecular pathogenesis of cervical and other HPV-associated malignancies. Functional diversity in the E6 and E7 oncoproteins of the HPVs is likely to be at least partly responsible for the observed differences between the oncogenic potentials of the various HPV types.

Recent studies in cell-free systems demonstrate that E6 proteins of high-risk HPV types, unlike E6 proteins of low-risk types, bind to and promote the degradation of p53 via the ubiquitin-dependent protease system (Scheffner *et al.*, 1990, 1993). One group was unable to demonstrate binding of p53 by the low-risk E6 proteins (Werness *et al.*, 1990), while another reported binding of both high- and low-risk E6 to p53, although the latter bound with substantially lower affinity (Crook *et al.*, 1991). Other investigators have also identified low-affinity binding of p53 to GST–11E6 fusion proteins and have shown that HPV11-E6 expression leads to partial inhibition of site-specific DNA binding by p53 (Lechner and Laimins, 1994). Another type of low-risk (HPV6) E6 has also been shown to modulate transcriptional regulatory functions by p53 (Crook *et al.*, 1994). Previous analyses of HPV E6 oncoproteins suggest that the p53 degradation and binding functions may be encoded by distinct DNA sequences (Crook *et al.*, 1991). Furthermore, the sequences encoding the degradation function are conserved among viruses according to their classification into high- and low-risk types. These findings suggest a possible mechanism to explain at least some of the differences in the oncogenic potential of E6 proteins encoded by low-risk versus high-risk HPV types.

To investigate the functional consequences of mutations in the regions of E6 proposed to be important for p53 binding and degradation in *in vivo* as well as *in vitro* assays, we generated three high-risk (HPV16) E6 mutants and one low-risk (HPV11) E6 mutant (Fig. 1). In HPV16E6ΔD, five amino acids are deleted in a region previously shown to interfere with the degradation of p53, but not with p53 binding. HPV16E6ΔB contains a deletion of six amino acids in a region shown to be necessary for p53 binding. Two E6 constructs with more subtle mutations were made (HPV16E6HL and HPV11E6LH) which exchanged amino acids in a putative p53 binding domain from high- to low-risk types and vice versa. These exchange mutants allowed us to determine whether this region alone would be sufficient to mediate p53 binding.

In the *in vitro* binding and degradation assays, complete correlation was found between the capacity to bind p53 and the capacity to enhance the degradation of p53. Only wild-type HPV16-E6 bound specifically to a GST–p53 fusion protein and directed the degradation of p53. None of the mutant E6 proteins showed p53 binding or enhancement of p53 degradation. Thus, in contrast to previous reports, we were unable to separate p53 binding and degradation domains with the mutant E6 forms that we generated. These differences could be explained by small differences between the constructs used or possibly by the assays employed. For example, deletion of an additional arginine at position 8 in HPV16E6ΔD might be responsible for the differences observed between this clone and a previously described E6 mutant with a deletion of amino acids 9 through 13 (Crook *et al.*, 1991). In agreement with our findings, a similar mutant of E6, with deletion of amino acids 9 through 13, failed to bind to, or enhance the degradation of p53 (Foster *et al.*, 1994).

The HPV16E6ΔB protein was functionally similar to proteins with other deletions in the region between amino acids 106 and 115. Interestingly, the mutant HPV16E6HL demonstrated that the ability of HPV16-E6 to bind and degrade p53 or abrogate p53-dependent G_1 arrest can be disrupted by rather subtle alterations exchanging a small region of HPV16-E6 to sequences found in low-risk types. Replacing this same region in a low-risk type E6 with the high-risk sequence does not restore p53 binding and/or degradation. Thus, the region between amino acids 106 and 115 appears to be necessary, but not sufficient, for p53 binding and degradation. A mutant with a deletion partly overlapping ours (amino acids 111 through 115) was also defective in p53 binding and did not enhance p53 degradation *in vitro* (Foster *et*

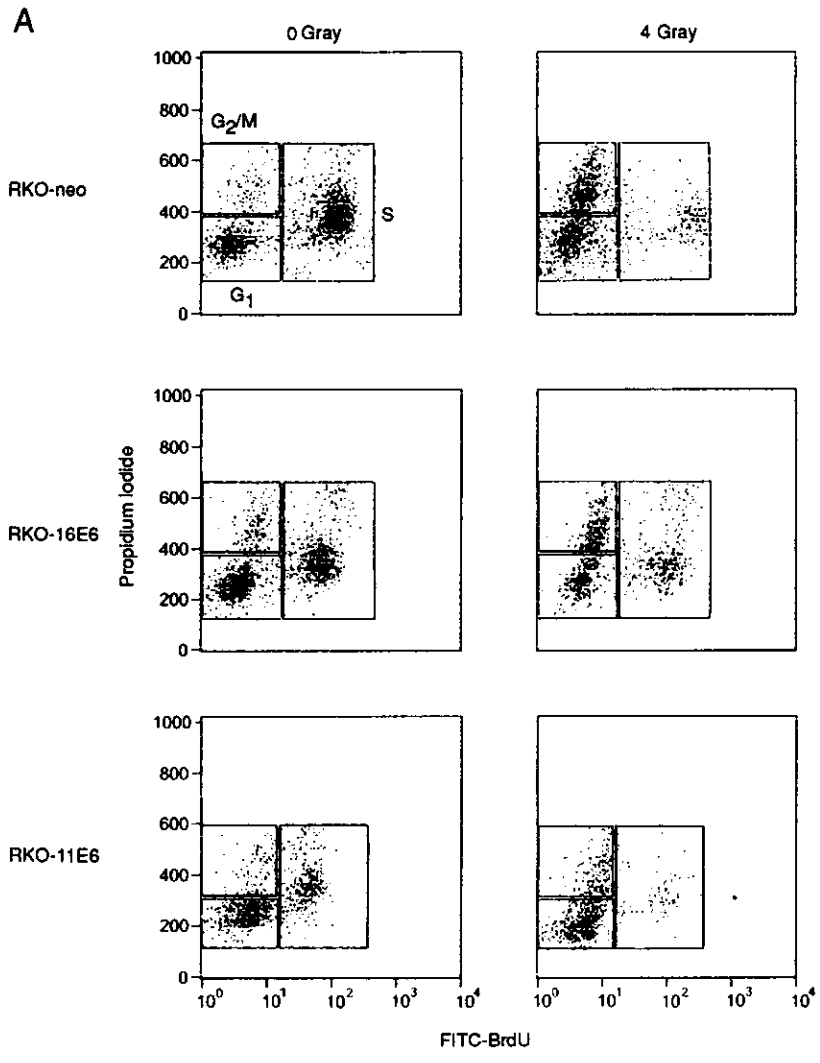

FIG. 6. Flow cytometric cell cycle analysis of HPV E6-transfected RKO cell lines. (A) Cell cycle distribution of RKO-neo (RCneo-1, transfected with vector alone), RKO-16E6 (RC10-1, expressing wild-type HPV16-E6), and RKO-11E6 (RC11-6, expressing wild-type HPV11-E6) 17 hr after either 0 or 4 Gy of γ-irradiation. G_1 arrest and associated depletion of S-phase cells are observed in RKO-neo and RKO-11E6 following irradiation. G_1 arrest is not observed in RKO-16E6. The p53-independent G_2 arrest in response to γ-irradiation remains intact in all cell lines tested. (B) Cell cycle distribution of untreated or γ-irradiated RKO parental cells (RKO and RCneo-1), RKO cells expressing HPV16-E6 (RC10-1, 10-2, 10-3), HPV11-E6 (RC11-6 and RC11L-2), HPV16E6ΔB (RC61-2, RC61-4), HPV16E6ΔD (RC73-5, RC73L-4), 16E6HL (RC33-2, RC33-9), and 11E6LH (RC41-5, RC41-9). Data are plotted as G_1/S ratios ± SE obtained from at least three independent measurements (except for RKO and RC10-2, two measurements each).

al., 1994). These findings suggest that the interaction between HPV E6 and p53, mediated by the cellular protein E6-AP, may involve larger regions within E6 or possibly secondary structures that can be disturbed by subtle mutations within the sequence. Accumulation of p53 after DNA damage is still intact in HPV11-E6-expressing cells, even in those that express very high levels of 11E6 mRNA (RC11-6), and these cells remain capable of DNA damage-induced G_1 cell cycle arrest.

The detection of HPV E6 proteins has historically been difficult due to the lack of high-affinity antibodies. Concurrent with the experiments described here, we attempted to generate antibodies to HPV16- and HPV11-E6. Although all of our E6-transfected cell lines express high levels of E6 mRNA, neither of these polyclonal antisera were capable of detecting E6 protein in immunoprecipitations or immunoblots using whole-cell lysates. The E6 expression constructs that we used might start their translation from the methionine in the E6 open reading frame that results in a protein of 151 amino acids (the natural start) and not from the upstream −7 codon that is sometimes used for *in vitro* protein expression (resulting in a protein of 158 amino acids). In previous studies, this 151-amino-acid E6 was undetectable by immunoprecipitation, in contrast to the longer 158-amino-acid form (Foster *et al.,* 1994). This may explain our failure to detect HPV16-E6 by immunoprecipitation using polyclonal antibodies to HPV16-E6 (our own and those kindly provided by Dr. D. Galloway). Furthermore, none of the mutant E6 proteins described here proved to be unstable in the *in vitro* p53 binding and degradation studies (Figs. 2 and 3). These results, together with the concordance between the p53 binding and degradation assays and the DNA damage response in RKO transfectants, strongly support the validity of the observed properties of the E6 variants described here.

Our observations suggest a plausible mechanism to explain at least some of the difference in oncogenic potential between low- and high-risk HPVs. The four mutant forms of E6 showed behavior similar to HPV11-E6; independent clones transfected with these constructs showed p53 accumulation and G_1 arrest following DNA damage, indicating that these mutant E6 proteins did not interfere with p53 function in living cells. The results obtained in RKO cells correlate closely with the observations made in the *in vitro* p53 binding and degradation assays. Furthermore, these studies clearly demonstrate that minor changes in the amino acid sequence of the E6 oncoprotein may have important functional consequences for cell cycle control and possibly other p53-mediated processes within the cell.

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