The Ability of Human Papillomavirus E6 Proteins To Target p53 for Degradation In Vivo Correlates with Their Ability To Abrogate Actinomycin D-Induced Growth Arrest

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Functional p53 protein is associated with the ability of cells to arrest in G₁ after DNA damage. The E6 protein of cancer-associated human papillomavirus type 16 (HPV-16) binds to p53 and targets its degradation through the ubiquitin pathway. To determine whether the ability of E6 to interact with p53 leads to a disruption of cell cycle control, mutated E6 proteins were tested for p53 binding and p53 degradation targeting in vitro, the ability to reduce intracellular p53 levels in vivo, and the ability to abrogate actinomycin D-induced growth arrest in human keratinocytes. Mutations scattered throughout the amino terminus, either zinc finger or the central region but not the carboxy terminus, severely reduced the ability of E6 to interact with p53. Expression of HPV-16 E6 or mutated E6 proteins that bound and targeted p53 for degradation in vitro sharply reduced the level of intracellular p53 induced by actinomycin D in human keratinocytes. A perfect correlation between the ability of E6 proteins to reduce the level of intracellular p53 and their ability to block actinomycin D-induced cellular growth arrest was observed. These results suggest that interaction with p53 is important for the ability of HPV E6 proteins to circumvent growth arrest.

In the last few years, the importance of the p53 protein in the development of cancer has become more clear. For example, the p53 gene is the most frequently mutated gene in human cancers (44), and in the case of Li-Fraumeni syndrome, germ line transmission of a mutated p53 gene is associated with the development of a variety of cancers during the lifetime of the individual (28, 42). One function of p53 that has been elucidated is its ability to act as a transcriptional activator by virtue of its ability to bind specific DNA sequences (2, 9, 10, 21, 36). More recently, the requirement for p53 in the growth arrest that occurs following cellular DNA damage has been elucidated (20, 23, 26). The ability to undergo growth arrest may allow time for the cells to complete the DNA repair process prior to entering S phase to avoid setting mutations permanently into the genome (15, 24). Cells lacking wild-type p53 do not display the appropriate G₁ arrest after DNA damage and exhibit high rates of gene amplification, one marker of genomic instability (27, 48).

The importance of p53 is underscored by the observation that several DNA tumor viruses encode proteins that interact with p53. High-risk-type human papillomaviruses (HPVs), which are associated with the development of cervical cancer (49), encode an E6 protein which binds to p53 and stimulates its degradation via the ubiquitin protein degradation pathway (40, 46). A 100-kDa protein, E6-AP, is involved in the p53 interaction and in conjunction with E6 acts as a ubiquitin-protein ligase (17, 18, 38). Low-risk HPV type 6 (HPV-6), which is not associated with cervical cancer, encodes an E6 that interacts with p53 weakly or not at all (4, 40, 46).

As predicted from its ability to bind and to target p53 for degradation in vitro, expression of high-risk HPV-16 E6 (16 E6) abrogates the transactivation function of p53 and the ability of cells to undergo p53-mediated growth arrest, while expression of HPV-6 E6 (6 E6) does not (5, 22, 30). These

results illustrate that 16 E6 can abrogate a function of the endogenously expressed cellular p53. In addition to its ability to interact with p53, E6 has transcriptional modulating activities which appear to be independent of any effect on p53 (4, 7).

16 E6 is 151 amino acids in length and contains four Cys-X-X-Cys motifs which may mediate zinc binding and the formation of two zinc finger structures (Fig. 1) (1, 12, 19). Although mapping of E6 domains which are important for the ability of E6 to interact with p53 has been reported, some discrepancy exists in the literature (4, 39, 40, 46). We have tested a number of HPV E6 proteins for the ability to bind and to target p53 for degradation and correlated these results with the ability of these proteins to block actinomycin D-induced growth arrest when expressed in primary human foreskin keratinocytes. Our results indicated that the ability of 16 E6 to bind and to target p53 for degradation in vitro is sensitive to mutations or small deletions in the amino terminus, either zinc finger, or the central region. 16 E6 proteins deleted in the carboxy-terminal region retained reduced but detectable activities. The ability of HPV E6 proteins to reduce the intracellular concentration of p53 correlated with their ability to abrogate actinomycin D-induced growth arrest.

MATERIALS AND METHODS

Plasmids. 6b and 16 E6 genes were subcloned into pBS(+) (Stratagene, La Jolla, Calif.) from pLXSN 6b E6 and pLXSN 16 E6 (13), respectively, and designated pBS 6 E6 and pBS 16 E6.

Mutated 16 E6 genes Δ 9-13, Δ 73-77, Δ 111-115, Δ 118-122, Δ 123-127, Δ 128-132, and 45Y/47Y/49H in plasmid pTZ18U or pTZ19U were provided by K. Vousden (St. Mary's Hospital Medical School, London, England) and have been described elsewhere (4). Our clone of the 16 E6 Δ 111-115 gene had an asparagine to lysine mutation at position 127 and is herein referred to as 16 E6 Δ 111-115/127K. Plasmids pSR α -E6C66G, -E6C136G, and -E6C66G/C136G were from T. Kanda (Na-

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FIG. 1. Amino acid sequence of 16 E6 showing various mutations. The arrow marks the point at which the 6/16 E6 chimeras were joined as described previously (4). The shaded regions indicate zinc finger motifs. Boxes indicate the positions of deletions; substitutions are indicated above the sequence. 16 E6 45Y/47Y/49H, 16 E6 Δ 9-13, 16 E6 Δ 73-77, 16 E6 Δ 78-82, 16 E6 Δ 111-115/127K, 16 E6 Δ 118-122, and 16 E6 Δ 128-132 were from K. Vousden (4); 16 E6 66G, 16 E6 136G, and 16 E6 66G/136G were from T. Kanda (19); 16 E6¹⁵¹ 8S/9A/10T was from P. Howley (30).

tional Institute of Health, Tokyo, Japan) (19). For in vitro transcription and translation, the E6 genes were subcloned into the *Hind*III site of pBS(+), and designated pBS-16E6 66G, -136G, and -66G/136G. Plasmids pJH26 (encoding 16 E6¹⁵¹ 8S/9A/10T) and pph53wt were obtained from P. M. Howley (Harvard Medical School, Boston, Mass.) (30, 46). Plasmid pph53wt was modified by inserting a cap-independent translation enhancer amplified by PCR from pCITE-1 (Novagen, Madison, Wis.) immediately upstream of the p53 initiation codon to create pph53wt-c. This modification did not alter the p53 initiation site.

Chimeric E6 genes, 6/16 E6 and 16/6 E6, were constructed as described previously (4). The 6/16 chimeric protein contains the N-terminal 60 amino acids from 6 E6 and the C-terminal 92 amino acids from 16 E6; the 16/6 chimeric protein contains the N-terminal 59 amino acids from 16 E6 (plus the upstream 7 amino acids from 16 E6 as described in Results) and the C-terminal 90 amino acids from 6 E6. For in vitro transcription and translation, the chimeric E6 genes were subcloned into pBS(+). The structures of the chimeric E6 genes were confirmed by DNA sequencing.

16 E6¹⁵¹, 16 E6¹⁵¹ Δ 135-151, 16 E6¹⁵¹ Δ 140-151, 16 E6¹⁵¹ Δ 140-151, and 16 E6*1 were created by PCR cloning strategies, cloned into pBS(+), and confirmed by DNA sequencing.

p53 degradation assays. The assay was performed as described previously (40). Briefly, 2 μ l each of in vitro-translated [³⁵S]cysteine-labeled p53 (rabbit reticulocyte lysate) and E6 (wheat germ extract) protein was mixed with 4 μ l of additional rabbit reticulocyte lysate, 12 μ l of water, and 20 μ l of 2 $\times$ degradation buffer containing 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 6 mM dithiothreitol. After removal of an aliquot for the zero time point, the reaction mixtures were incubated at 25°C. At various times, aliquots were removed and mixed with an equal volume of 2 $\times$ sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (37). The samples then were analyzed by SDS-PAGE and fluorography. The gels were quantitated by PhosphorImager scanning on a Molecular Dynamics 400A PhosphorImager with ImageQuant software (Molecular Dynamics, Sunny-

vale, Calif.). To compare the relative abilities of the various E6 proteins to target p53 for degradation, assays containing 1:2 serial dilutions of 16 E6 were performed and used as a standard curve. The degree of p53 degradation induced by each E6 protein was compared against an HPV-16 E6 control run in a parallel reaction and against the standard curve. The p53 degradation activity, relative to 16 E6, was calculated by noting the relative amount of each E6 protein and the degree of p53 degradation.

p53 binding assays. The assay was performed as described previously (46). Briefly, 10 μ l of unlabeled in vitro-translated p53 and 10 μ l of [³⁵S]cysteine-labeled in vitro-translated E6 were added to 20 μ l of cell lysis buffer containing 0.1 M Tris-HCl (pH 8.0), 0.1 M NaCl, and 1% Nonidet P40 and incubated on ice. After 2 to 3 h, the reactions were immunoprecipitated by using a monoclonal antibody against p53 (p53 Ab 1; Oncogene Science, Uniondale, N.Y.) and protein A-Sepharose beads (Pharmacia, Piscataway, N.J.) (37). After four washes in cell lysis buffer, bound proteins were released by adding 2 $\times$ SDS sample buffer. The samples then were analyzed by SDS-PAGE and fluorography. The gels were quantitated by PhosphorImager scanning as described above. To assess non-specific binding, labeled E6 proteins were combined with water-programmed rabbit reticulocyte lysate and similarly immunoprecipitated. The nonspecific binding was always subtracted from the p53-associated E6 in determining the extent of E6-p53 binding. The percentage of input E6 coimmunoprecipitated with p53 was calculated and expressed relative to 16 E6 binding.

Production of E6-expressing retroviruses. E6 genes were cloned into the retroviral vector pLXSN (34), and these constructs were used to produce amphotropic retroviruses (31). Briefly, the constructs were transfected by the calcium phosphate method into Psi-2 or PE501 cells (29, 34). Virus produced from these cells was used to infect PA317 or PG13 cells (32, 33). Individual G418-resistant clones were isolated and confirmed to carry the E6 gene by Southern blotting (37).

Metabolic labeling and immunoprecipitation of HPV E6.

TABLE 1. In vitro p53 binding and degradation activities of E6 proteins

HPV E6 protein	p53 binding (% of 16 E6 activity)	p53 degradation (% of 16 E6 activity)
16 E6	100	100
6 E6	— ^a	—
6/16 E6	—	—
16/6 E6	—	—
16 E6 ¹⁵¹	120	50
16 E6 ¹⁵¹ Δ2-12	—	NS ^b
16 E6 Δ9-13	—	—
16 E6 ¹⁵¹ 8S/9A/10T	—	—
16 E6 ¹⁵¹ 45V	29	25
16 E6 45Y/47Y/49H	—	—
16 E6 Δ73-77	—	—
16 E6 Δ78-82	—	—
16 E6 Δ111-115/127K	—	—
16 E6 Δ118-122	—	2
16 E6 Δ123-127	—	—
16 E6 Δ128-132	—	—
16 E6*I	NS	NS
16 E6 ¹⁵¹ Δ136-151	NS	NS
16 E6 ¹⁵¹ Δ140-151	35	25
16 E6 ¹⁵¹ Δ146-151	33	67
16 E6 66G	—	—
16 E6 136G	—	2
16 E6 66G/136G	—	—

^a —, no activity over background levels was detected.

^b NS, the E6 protein was not translated well enough or was not stable enough to evaluate.

Cultures of retrovirally infected keratinocytes were labeled with [³⁵S]cysteine (200 μCi/ml; Amersham, Arlington Heights, Ill.), and E6 was immunoprecipitated with rabbit antiserum raised against E6 (11) and protein A-Sepharose beads by standard procedures (37).

p53 immunoblots. Subconfluent cultures of retrovirally infected keratinocytes expressing various HPV E6 genes were treated with 0.5 nM actinomycin D. After 24 h, the cells were rinsed with phosphate-buffered saline, lysed in 1× SDS sample buffer, and sonicated. The relative protein content of each sample was checked by SDS-PAGE followed by staining with Coomassie blue and densitometry. Aliquots were analyzed by SDS-PAGE followed by immunoblotting with anti-p53 antibody (p53 Ab 6; Oncogene Science). Detection was achieved by chemiluminescence (Renaissance; Du Pont NEN, Boston, Mass.).

Growth arrest analysis. Primary human foreskin keratinocytes were maintained in keratinocyte SFM medium (Gibco-BRL, Gaithersburg, Md.). Low-passage-number keratinocytes were infected with pLXSN-based retroviruses (13, 34) containing various E6 genes and selected with 50 μg of G418 per ml. As soon as sufficient numbers of G418-resistant (i.e., E6-expressing) cells were obtained, subconfluent cultures of retrovirally infected keratinocytes expressing various HPV E6 genes were treated with 0.5 nM actinomycin D or left untreated. After 24 h, the cells were treated with 10 μM bromodeoxyuridine (BrdU) for 4 h. The cells then were fixed in ethanol, and the nuclei were stained with anti-BrdU-fluorescein isothiocyanate conjugate antibody (Becton Dickinson, San Jose, Calif.) and propidium iodide as described previously (47), with minor modifications. The stained nuclei were analyzed on a Becton Dickinson FACScan; 10,000 events were analyzed. Quantitation of the results was performed by using Reproman software (TrueFacts Software, Seattle, Wash.). Doublet discrimination was done by gating plots of

FIG. 2. In vitro p53 interaction between HPV E6 proteins and p53. (A) p53 binding assays. Lanes: +, E6 coimmunoprecipitated in the presence of p53; —, E6 coimmunoprecipitated in the presence of water-primed lysate; I, an aliquot run without immunoprecipitation to verify the amount of E6 in the reaction. (B) In vitro degradation of p53 induced by HPV E6 proteins. Samples of the reaction were taken at time zero (lane 1), 3 h (lane 2), or 14 to 18 h (lane 3).

propidium iodide fluorescence (area versus width), and percentages were calculated from plots, as shown in Fig. 5.

RESULTS

p53 binding and degradation by HPV E6 proteins in vitro.

To examine the interaction between E6 and p53, E6 proteins (Fig. 1) and p53 were translated in vitro and tested in binding and degradation assays. Binding to p53 was assessed by coimmunoprecipitation of [³⁵S]cysteine-labeled E6 with unlabeled p53. In the p53 degradation assay, both E6 and p53 were labeled. The results were visualized by SDS-PAGE and fluorography. Table 1 shows a summary of the p53 binding and degradation results, and Fig. 2 shows specific examples. In agreement with previous studies (4, 40, 46), 16 E6 bound to and stimulated degradation of p53. On average, 3.4% of 16 E6 was coimmunoprecipitated with p53 in the binding assay. Typically, 96% of p53 was degraded after 14 to 18 h in the presence of 16 E6 in the degradation assay. Neither p53 binding nor degradation was detected with 6 E6.

To begin to localize regions of 16 E6 that are important for its interaction with p53, two chimeric E6 proteins similar to those described previously (4) were tested for p53 binding and degradation functions. The 6/16 chimeric protein contains the N-terminal 60 amino acids from 6 E6 and the C-terminal 92 amino acids from 16 E6, while the 16/6 chimeric protein contains the N-terminal 59 amino acids from 16 E6 and the C-terminal 90 amino acids from 6 E6. Both chimeric E6 proteins, 6/16 and 16/6, were defective for p53 binding and degradation (Table 1).

The 16 E6 clone used here and in other studies (4, 40, 46) contains two methionine codons near the 5' end of the open reading frame and yields two polypeptides corresponding to 158- and 151-amino-acid products, suggesting that both initiation codons are used in the in vitro transcription and translation system. Based on RNA mapping studies which place the 5' end of the E6-containing mRNA at position 97 (41) and alignments with other HPV E6 genes, the second methionine codon represents the natural start site for translation of 16 E6. Thus, in HPV-containing cells, a 151-amino-acid 16 E6 protein

is predicted. To determine whether the upstream 7 amino acids affect p53 binding or degradation functions of 16 E6, an E6 construct lacking the upstream initiation codon and thus coding only for the 151-amino-acid 16 E6 product (16 E6¹⁵¹) was tested. The 16 E6¹⁵¹ protein bound to p53 at levels slightly higher (120%), and induced degradation of p53 at levels lower (50%), than the 158/151 16 E6 commonly used. This finding indicates that the upstream seven amino acids of 16 E6 are not essential for its ability to bind and to target p53 for degradation, although they may influence the p53 interaction somewhat.

Further deletion of residues at the amino-terminal end, 16 E6¹⁵¹ Δ2-12, gave a protein that was defective for p53 binding, indicating that amino-terminal amino acid sequences are important for this activity. However, whether this protein induced p53 degradation was unclear, as 16 E6¹⁵¹ Δ2-12 was unstable under the p53 degradation assay conditions. 16 E6 proteins more extensively deleted from the amino-terminal end either failed to bind p53 or failed to yield enough protein from *in vitro* translation to evaluate (not shown). A 16 E6 protein with a five-amino-acid deletion within the amino-terminal portion (16 E6 Δ9-13) failed to bind or to stimulate p53 degradation at detectable levels. A 16 E6 protein substituted at amino acid residues 8, 9, and 10 with the corresponding amino acids of the 6 E6 protein, 16 E6¹⁵¹ 8S/9A/10T, also failed to bind p53 or target p53 for degradation, in agreement with a previous report (30). These results indicate that the amino-terminal portion of 16 E6 is important for its ability to interact with p53.

Since the amino-terminal sequences of 16 E6 were important for p53 interaction, the carboxy-terminal sequences were examined. The 16 E6¹⁵¹ Δ146-151 protein retained p53 binding and degradation activities, although less than 16 E6 (Table 1; Fig. 2). The 16 E6¹⁵¹ Δ140-151 protein also showed unambiguous but reduced activity in both p53 binding and degradation assays. The 16 E6¹⁵¹ Δ136-151 deletion, which disrupts the second zinc finger motif, failed to yield enough protein from *in vitro* translation to evaluate, as did a construct expressing the 16 E6*1 product, which is potentially generated by splicing of the 16 E6 RNA *in vivo* and lacks the carboxy-terminal two-thirds of 16 E6. These results indicate that the carboxy-terminal portion of 16 E6 that extends past the second zinc finger is not essential for its ability to bind and to target p53 for degradation, although deletion of this region does reduce its ability to bind and to target p53 for degradation.

A number of mutated 16 E6 proteins with either substitutions or small deletions within the two zinc fingers or the central region were tested for the ability to bind p53 and to target p53 for degradation (Table 1). One protein, 16 E6¹⁵¹ 45V, which carries a phenylalanine-to-valine substitution at amino acid 45 within the first zinc finger, retained a reduced ability to bind p53 and promote p53 degradation. In contrast, no p53 binding or degradation was detected for a triple substitution, 16 E6 45Y/47Y/49H. Two proteins with five-amino-acid deletions within the central region, 16 E6 Δ73-77 and 16 E6 Δ78-82, also were defective for p53 binding and degradation. Three proteins with deletions in the second zinc finger, 16 E6 Δ111-115/127K, Δ118-122, and Δ128-132 were defective for both p53 binding and degradation, although 16 E6 Δ118-122 showed a very weak ability to target p53 for degradation, about 2% of 16 E6 activity (Fig. 2). The detection of weak p53 degradation activity without concomitant detection of weak p53 binding suggests that the p53 degradation assay may be more sensitive than the p53 binding assay. As can be seen in Fig. 2, some of the mutant E6 proteins gave slightly elevated backgrounds, but since the amounts were similar with

FIG. 3. RIP of E6 proteins. Extracts were prepared from [³⁵S]cysteine-labeled cells, and E6 proteins were immunoprecipitated with rabbit antiserum (9). The original film was scanned with a Sharp JX-325 scanner and Adobe Photoshop software. The image was printed on a Tektronix Phaser II SDX dye sublimation printer.

and without added p53, we judged these to be negative for p53 binding.

Three 16 E6 constructs with glycine substitutions in the Cys-X-X-Cys zinc finger motifs, 16 E6 66G, 16 E6 136G, and 16 E6 66G/136G, also were tested. Again, these E6 proteins were defective for p53 binding and degradation, although very low p53 degradation activity (2% of HPV-16 E6 activity) was detected for the 16 E6 136G protein. Thus, mutations or small deletions which affect either zinc finger or the central region produce a sharp decrease in the ability of E6 to interact with p53, suggesting that these regions, in addition to the amino-terminal region, are important for p53 interaction.

Construction and characterization of retroviruses expressing mutated HPV E6 proteins. To introduce E6 genes into human keratinocytes, a subset of the mutated E6 genes was subcloned into the retroviral vector pLXS (34). Ecotropic

TABLE 2. *In vivo* effects of E6 expression: p53 degradation and abrogation of growth arrest

Construct	E6 detection in vivo ^a	p53 degradation in vivo	Abrogation of growth arrest ^b in expt:				
			I	II	III	IV	V
Vector	—	—	0.03	0.15	0.08	0.29	0.26
16 E6	+ ^c	+	1.02	1.21	1.16	1.14	1.13
16 E6 ¹⁵¹	—	+			1.03		1.12
6 E6	+ ^c	—	0.06	0.17			
16 E6 ¹⁵¹ 8S/9A/10T	—	—		0.41			0.35
16 E6 Δ9-13	—	—			0.06		0.24
16 E6 45Y/47Y/49H	+	—		0.14			0.10
16 E6 Δ73-77	+	—				0.10	0.18
16 E6 Δ78-82	+	—			0.08		0.11
16 E6 Δ111-115/127K	+/-	—			0.03		0.20
16 E6 Δ118-122	+	+			1.14		1.05
16 E6 Δ123-127	+	—			0.07		0.18
16 E6 Δ128-132	+	—			0.08		0.15
16 E6 ¹⁵¹ Δ136-151	—	—			0.06		0.08
16 E6 ¹⁵¹ Δ140-151	+/-	+			1.20		0.99
16 E6 ¹⁵¹ Δ146-151	—	+			1.03		1.02
6/16 E6	—	—	0.05				0.25
16/6 E6	+	—	0.16				0.32

^a +, clearly detected; +/-, ambiguous; —, not detected.

^b Values were calculated as percentage of S-phase cells in the presence of actinomycin D divided by percentage of S-phase cells in the absence of actinomycin D. Values close to 1 indicate abrogation of growth arrest (highlighted by boldface type); values close to zero indicate growth arrest induced by actinomycin D.

^c Retroviruses previously characterized (13).

FIG. 4. p53 protein levels in actinomycin D-treated primary human foreskin keratinocytes expressing different HPV E6 proteins. The label for each lane refers to the E6 protein expressed in the cells with deletions or substitutions as indicated (see Fig. 1). Δ 111-115 also carries the 127K mutation. LXSN, vector-infected cells. The original film was processed for publication as in Fig. 3.

retroviruses, produced by transfecting the constructs into Psi-2 or PE501 packaging cells, were used to infect PA317 or PG13 packaging cells from which amphotropic E6-expressing retroviruses were produced. The 16 E6- and 6 E6-expressing retroviruses have been described previously (13).

To confirm that the retroviruses were capable of conferring the expression of E6 protein, the amphotropic retroviruses were used to infect human keratinocytes and/or C33A cells. Following selection with G418, the cells were metabolically labeled with [35 S]cysteine and extracts were prepared for use in radioimmunoprecipitations (RIP). Although E6 protein historically has been difficult to detect because of the lack of high-avidity antibodies, E6 was detected by RIP in most of the E6-expressing cell lines. Figure 3 shows several examples of E6 precipitations, and Table 2 summarizes the results. The following E6 proteins were detected by RIP in infected cells: 16 E6 (standard construct), 16 E6 Δ 45Y/47Y/49H, 16 E6 Δ 73-77, 16 E6 Δ 78-82, 16 E6 Δ 118-122, 16 E6 Δ 123-127, 16 E6 Δ 128-132, and 16/6 E6. Detection of the remaining E6 proteins was ambiguous; however, three of these (16 E6 151 , 16 E6 151 Δ 140-151, and 16 E6 151 Δ 146-151) had a pronounced effect on p53 levels and growth arrest (see below). Therefore, it is apparent that these proteins are indeed expressed but not readily detected by RIP. E6 proteins 16 E6 151 8S/9A/10T, 16 E6 Δ 9-13, 16 E6 Δ 111-115/127K, and 6/16 E6 were not clearly detected by RIP, although these proteins were stable when translated in vitro. Whether these proteins were translated less efficiently, were less stable, or simply were less readily detectable is not clear. 16 E6 Δ 136-151 was not detected by RIP and was not translated well in vitro; thus, it may be an unstable protein.

p53 protein levels in cells expressing HPV E6 proteins. One effect of DNA damage is to increase the level of p53 protein in the cell by a posttranscriptional mechanism (20). Low levels of actinomycin D induce DNA strand breaks by interfering with the action of cellular topoisomerases (43, 45). To test the ability of E6 proteins to target p53 for degradation under more physiologically relevant conditions, various E6 genes were introduced into primary human foreskin keratinocytes by retrovirus-mediated gene transfer (13, 34), and the relative amounts of p53 induced in the E6-expressing keratinocytes exposed to actinomycin D were examined. Previous results demonstrate that expression of 16 E6 in keratinocytes increases the degradation rate of p53 (16) and reduces the baseline and induced levels of p53 protein (5, 6). Cells were

treated with 0.5 nM actinomycin D for 24 h, after which total cell extracts were prepared and analyzed by immunoblotting for p53. The results of this analysis are shown in Fig. 4. A clear p53 signal was detected in vector-infected cells, while cells expressing E6 proteins that targeted p53 for degradation in vitro (16 E6, 16 E6 151 , 16 E6 151 Δ 140-151, and 16 E6 151 Δ 146-151) exhibited only trace amounts of p53 protein. Cells expressing 16 E6 Δ 118-122 had a sharply reduced intracellular level of p53 protein, although the reduction was not as pronounced. Interestingly, 16 E6 Δ 118-122 had only a very weak ability to target p53 for degradation in vitro. We interpret this result to indicate that the 16 E6 Δ 118-122 protein interacts with p53 more strongly in the intracellular environment or that a very weak p53 interaction is sufficient to reduce p53 levels in vivo. Other mutated E6 proteins that failed to bind and to target p53 for degradation in vitro did not produce a distinct reduction in intracellular p53. Thus, of the 17 E6 proteins tested for the ability to target p53 for degradation in vivo, 16 gave results that were entirely consistent with their ability to target p53 for degradation in vitro.

Abrogation of actinomycin D-induced growth arrest by HPV E6 proteins. Low levels of actinomycin D induce DNA strand breaks by interfering with the action of cellular topoisomerases (43, 45). Growth arrest, predominantly at G₁, ensues (3). The G₁ checkpoint requires a functional p53 protein (23, 27, 48). To examine the consequences of HPV E6-p53 interaction in vivo, we tested the ability of E6 to abrogate p53-mediated growth arrest after DNA damage. Various E6 genes were introduced into primary human foreskin keratinocytes by retrovirus-mediated gene transfer as described above and treated with 0.5 nM actinomycin D as a DNA-damaging agent or left untreated. After 24 h, the cells were treated with BrdU for 4 h and then stained with anti-BrdU-fluorescein isothiocyanate antibody to label newly replicated DNA and propidium iodide to label total DNA. The cells then were analyzed by fluorescence-activated cell sorting to identify cells in G₁, S, and G₂.

The results of this analysis are presented in Table 2, and examples of the plots are shown in Fig. 5. Keratinocytes infected with the pLXSN vector alone underwent growth arrest in response to actinomycin D treatment. The most obvious and consistent observation was a distinct decrease (varying from 70 to 97%) in the percentage of S-phase cells. As predicted by its ability to bind and to target p53 for degradation in vitro, 16 E6 effectively abrogated p53-mediated growth arrest in human keratinocytes. In contrast, 6 E6, which did not detectably interact with p53 in vitro, did not affect growth arrest. These results are in agreement with recent reports (5, 22).

Those E6 proteins which stimulated p53 degradation in vitro and sharply decreased the level of p53 in vivo also abrogated p53-mediated growth arrest in keratinocytes. These included 16 E6 151 , 16 E6 151 Δ 140-151, and 16 E6 151 Δ 146-151. The 16 E6 Δ 118-122 protein, which showed a very weak ability to target p53 for degradation in vitro but reduced the intracellular level of p53 in vivo, abrogated p53-mediated growth arrest as well as did 16 E6. Other mutated 16 E6 proteins that did not detectably interact with p53 in vitro and failed to reduce the intracellular level of p53 did not abrogate p53-mediated growth arrest. These included 16 E6 Δ 46Y/47Y/49H, 16 E6 Δ 73-77, 16 E6 Δ 78-82, 16 E6 Δ 111-115/127K, 16 E6 Δ 123-127, 16 E6 Δ 128-132, and 16/6 E6. E6 proteins 16 E6 151 8S/9A/10T, 16 E6 Δ 9-13, 16 E6 Δ 111-115/127K, and 16 E6 Δ 136-151 and the 6/16 E6 chimera also failed to block growth arrest; however, we were unable to confirm expression of these proteins. It should be noted that 16 E6 151 8S/9A/10T and 16/6 E6 gave a weak growth arrest abrogation in one experiment

FIG. 5. Actinomycin D-induced growth arrest of normal human keratinocytes expressing HPV E6 genes. The E6 genes are indicated at the left (see Fig. 1). LXSXN, vector-infected cells. For each plot, the left-hand box represents G₁- and G₀-phase cells and the right-hand box represents G₂- and M-phase cells. The remaining cells were considered S-phase cells.

but growth arrest was similar to that for the vector control in another experiment. These results demonstrate a strong correlation between the ability of mutated E6 proteins to reduce the intracellular level of p53 and the ability to abrogate growth arrest following DNA damage (Table 2).

DISCUSSION

The requirement for p53 in the G₁ growth arrest of cells following DNA damage provides a useful system for testing the ability of E6 proteins to interfere with a normal function of the endogenous p53 tumor suppressor protein in primary cells. We have tested the ability of a panel of mutated E6 proteins to interact with p53 in vitro and in vivo. All of the E6 proteins tested in this system that bound to and targeted p53 for

degradation in vitro were able to dramatically reduce the level of intracellular p53 and abrogate p53-mediated growth arrest in vivo. These included HPV-16 E6, 16 E6¹⁵¹, 16 E6¹⁵¹ Δ140-151, and 16 E6¹⁵¹ Δ146-151. Although some differences were noted in the ability of these proteins to interact with p53 in vitro, all retained wild-type ability to abrogate p53-mediated growth arrest in vivo. One explanation for these results is that a certain threshold level of p53 is required in order to trigger a growth arrest response. If the presence of E6 reduces the concentration of p53 to below the threshold level required to induce growth arrest, the cells would continue to cycle.

The E6 proteins that failed to bind and to target p53 for degradation in vitro also failed to reduce the level of intracellular p53 and did not abrogate p53-mediated growth arrest in vivo. These included HPV-6 E6, 16 E6¹⁵¹ 8S/9A/10T, 16 E6 Δ9-13, 16 E6 46Y/47Y/49H, 16 E6 Δ73-77, 16 E6 Δ78-82, 16 E6 Δ111-115/127K, 16 E6 Δ123-127, 16 E6 Δ128-132, 6/16 E6, and 16/6 E6. One exception was 16 E6 Δ118-122, for which no p53 binding and only a very weak ability to target p53 for degradation (2% of the 16 E6 level) was detected, and yet this protein reduced the level of intracellular p53 and retained the ability to completely abrogate p53-mediated growth arrest. One explanation for these results is that the 16 E6 Δ118-122 protein weakly interacts with p53 and this is sufficient to interfere with p53 function or that the weak p53 interaction observed in vitro is stabilized by cellular factors in vivo. For example, if 16 E6 Δ118-122 failed to fold properly in vitro, correct folding may be stabilized in an intracellular environment, thus allowing the protein to interact more efficiently with p53. Although it is formally possible that 16 E6 Δ118-122 abrogated growth arrest by a mechanism not involving direct p53 interaction, we consider this less likely than the former explanations primarily because expression of 16 E6 Δ118-122 reduced the level of intracellular p53, an effect that most likely involves some physical association with p53.

At present, no linear amino acid sequence that convincingly distinguishes the high-risk from the low-risk HPV E6 proteins has been presented. Therefore, it is reasonable to propose that the structural features required for p53 binding and targeting of p53 for degradation by E6 proteins arise through higher-order conformations. The data presented in this report support such a hypothesis since most of the altered HPV-16 E6 proteins were severely reduced in their ability to bind p53 and to target p53 for degradation. Mutations or small deletions within the amino-terminal region, either zinc finger, or the central region resulted in a dramatic reduction in the ability of 16 E6 to bind p53 and to target p53 for degradation. 16 E6 proteins deleted in the carboxy-terminal region, on the other hand, retained marked p53 binding and degradation activities, although reduced compared with HPV-16 E6 activities. Thus, the integrity of the amino-terminal region, both zinc fingers, and the central region may be necessary to maintain proper E6 protein structure.

In addition to the possible effects of mutations on the tertiary structure of E6 is the complication of the 100-kDa E6-AP which is required for stable E6-p53 binding. It is not known how E6-AP acts to facilitate p53-E6 binding except that E6-AP associates with E6 in the absence of p53 but not vice versa (17). It is possible that some mutations in E6 affect E6-AP interactions while others affect interactions with p53. In either case, E6-p53 binding would be disrupted.

Other studies of the interaction of mutated E6 proteins with p53 have produced different results. For example, Crook et al. (4) detected strong p53 binding without concomitant p53 degradation for 16 E6 Δ9-13 and 16 E6 46Y/47Y/49H in vitro and thus concluded that the p53 binding and degradation

targeting functions of E6 were separable. Lechner et al. (25) reported that 16 E6 46Y/47Y/49H inhibited p53-mediated transcriptional repression in a transient expression assay and concluded that E6 binding to p53 was sufficient for this inhibition. In our experiments, we were unable to detect p53 binding in vitro by either 16 E6 Δ 9-13 or 16 E6 46Y/47Y/49H protein. Furthermore, 16 E6 46Y/47Y/49H failed to abrogate p53-mediated growth arrest when expressed in keratinocytes.

There are several differences between the experiments of Lechner et al. (25) and the experiments described here. In the former study, the ability of E6 to inhibit p53-mediated transcriptional repression was assessed, while in this report, the ability of E6 to block p53-mediated growth arrest was assessed. Although the exact mechanism underlying growth arrest has not been elucidated, it may involve the p53-mediated transactivation of genes like p21^{Cip1}, which acts as an inhibitor of cyclin-dependent kinases (8, 14, 35). Additionally, in the former experiments, both E6 and p53 were introduced by transfection, which likely introduces multiple copies of both genes. In this study, E6 was introduced by retroviral infection, with most cells receiving one copy, and the cells contained only the endogenous p53 genes. Thus, the levels of E6 and p53 expression are likely to be much higher in the former experiments than in this study.

On the basis of the frequency of p53 mutations found in human cancers and the role of p53 in the control of cellular proliferation after DNA damage, the elimination of p53 function by 16 E6 has important implications for the development of cancer. The lack of wild-type p53 may permit genomic instability by disabling a biochemical pathway that allows cells to pause after DNA damage to repair their DNA, or to accumulate sufficient DNA precursors, before S phase (15, 24, 27, 48). In this model, lack of p53 may allow genetic damage to become set into the genome and, over a period of time, would increase the likelihood that immortal or tumorigenic cells will emerge. The crisis stage in cellular senescence is a time when the genome may be particularly unstable. In primary human keratinocytes, no gross genetic abnormalities were observed in 16 E6-expressing cells until after crisis (22a). Thus, inactivation of p53 by E6 may contribute to immortalization by preventing the cells from arresting in response to the genomic instability that occurs during senescence. Clones of cells in which random genetic damage has conferred a growth advantage may then emerge.

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REFERENCES

- Barbosa, M. S., D. R. Lowy, and J. T. Schiller. 1989. Papillomavirus polypeptides E6 and E7 are zinc-binding proteins. *J. Virol.* **63**:1404–1407.
- Bargonetti, J., P. N. Friedman, S. E. Kern, B. Vogelstein, and C. Prives. 1991. Wild-type but not mutant p53 immunopurified proteins bind to sequences adjacent to the SV40 origin of replication. *Cell* **65**:1083–1091.
- Craig, R. W., O. S. Frankfurt, H. Sakagami, K. Takeda, and A. Bloch. 1984. Macromolecular and cell cycle effects of different classes of agents inducing the maturation of human myeloblastic leukemia (ML-1) cells. *Cancer Res.* **44**:2421–2429.
- Crook, T., J. A. Tidy, and K. H. Vousden. 1991. Degradation of p53 can be targeted by HPV E6 sequences distinct from those required for p53 binding and transactivation. *Cell* **67**:547–556.
- Demers, G. W., S. A. Foster, C. L. Halbert, and D. A. Galloway. 1994. Growth arrest by induction of p53 in DNA damaged keratinocytes is bypassed by HPV 16 E7. *Proc. Natl. Acad. Sci. USA* **91**:4382–4386.
- Demers, G. W., C. L. Halbert, and D. A. Galloway. 1994. Elevated wild-type p53 protein levels in human epithelial cell lines immortalized by the human papillomavirus type 16 E7 gene. *Virology* **198**:169–174.
- Desaintes, C., S. Hallez, P. Van Alphen, and A. Burny. 1992. Transcriptional activation of several heterologous promoters by the E6 protein of human papillomavirus type 16. *J. Virol.* **66**:325–333.
- Eldeiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, W. E. Mercer, K. W. Kinzler, and B. Vogelstein. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell* **75**:817–825.
- Farmer, G., J. Bargonetti, H. Zhu, P. Freidman, R. Prywes, and C. Prives. 1992. Wild-type p53 activates transcription *in vitro*. *Nature (London)* **358**:83–86.
- Fields, S., and S. K. Jang. 1990. Presence of a potent transcription activating sequence in the p53 protein. *Science* **249**:1046–1049.
- Firzlaff, J. M., C. N. L. Hsia, C. L. Halbert, S. A. Jenison, and D. A. Galloway. 1987. Polyclonal antibodies to human papillomavirus type 6b and type 16 bacterially expressed fusion proteins. *Cancer Cells* **5**:105–113.
- Grossman, S., and L. A. Laimins. 1989. E6 protein of human papillomavirus type 18 binds zinc. *Oncogene* **4**:1089–1093.
- Halbert, C. L., G. W. Demers, and D. A. Galloway. 1991. The E7 gene of human papillomavirus type 16 is sufficient for immortalization of human epithelial cells. *J. Virol.* **65**:473–478.
- Harper, J. W., G. R. Adami, N. Wei, K. Keyomarsi, and S. J. Elledge. 1993. The p21 CDK-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* **75**:805–816.
- Hartwell, L. 1992. Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. *Cell* **71**:543–546.
- Hubbert, N. L., S. A. Sedman, and J. T. Schiller. 1992. Human papillomavirus type 16 E6 increases the degradation rate of p53 in human keratinocytes. *J. Virol.* **66**:6237–6241.
- Huibregtse, J. M., M. Scheffner, and P. M. Howley. 1991. A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18. *EMBO J.* **10**:4129–4135.
- Huibregtse, J. M., M. Scheffner, and P. M. Howley. 1993. Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. *Mol. Cell. Biol.* **13**:775–784.
- Kanda, T., S. Watanabe, S. Zanma, H. Sato, A. Furuno, and K. Yoshike. 1991. Human papillomavirus type 16 E6 proteins with glycine substitution for cysteine in the metal-binding motif. *Virology* **185**:536–543.
- Kastan, M. B., O. Onyekwere, D. Sidransky, B. Vogelstein, and R. W. Craig. 1991. Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.* **51**:6304–6311.
- Kern, S. E., K. W. Kinzler, A. Bruskini, D. Jarosz, P. Friedman, C. Prives, and B. Vogelstein. 1991. Identification of p53 as a sequence-specific DNA-binding protein. *Science* **252**:1708–1711.
- Kessis, T. D., R. J. Slebos, R. J. Nelson, M. B. Kastan, B. S. Plunkett, S. M. Han, A. T. Lorincz, L. Hedrick, and K. R. Cho. 1993. Human papillomavirus 16 E6 expression disrupts p53-mediated cellular response to DNA damage. *Proc. Natl. Acad. Sci. USA* **90**:3988–3992.
- Klingelhutz, A. J., S. A. Barber, P. P. Smith, K. Dyer, and J. K. McDougall. 1994. Restoration of telomeres in human papillomavirus-immortalized human anogenital epithelial cells. *Mol. Cell. Biol.* **14**:961–969.
- Kuerbitz, S. J., B. S. Plunkett, W. V. Walsh, and M. B. Kastan. 1992. Wild-type p53 is a cell cycle checkpoint determinant follow-

- ing irradiation. *Proc. Natl. Acad. Sci. USA* **89**:7491–7495.
24. Lane, D. P. 1992. p53, guardian of the genome. *Nature* (London) **358**:15–16.
 25. Lechner, M. S., D. H. Mack, A. B. Finicle, T. Crook, K. H. Vousden, and L. A. Laimins. 1992. Human papillomavirus E6 proteins bind p53 in vivo and abrogate p53-mediated repression of transcription. *EMBO J.* **11**:3045–3052.
 26. Lin, D., M. T. Shields, S. J. Ullrich, E. Appella, and W. E. Mercer. 1992. Growth arrest induced by wild-type p53 protein blocks cells prior to or near the restriction point in late G₁ phase. *Proc. Natl. Acad. Sci. USA* **89**:9210–9214.
 27. Livingstone, L. R., A. White, J. Sprouse, E. Livanos, T. Jacks, and T. D. Tlsty. 1992. Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. *Cell* **70**:923–935.
 28. Malkin, D., F. P. Li, L. C. Strong, J. F. Fraumeni, Jr., C. E. Nelson, D. H. Kim, J. Kassei, M. A. Gryka, F. Z. Bischoff, M. A. Tainsky, and S. H. Friend. 1990. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science* **250**:1233–1238.
 29. Mann, R., R. C. Mulligan, and D. Baltimore. 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* **33**:153–159.
 30. Mietz, J. A., T. Unger, J. M. Huibregtse, and P. M. Howley. 1992. The transcriptional transactivation function of wild-type p53 is inhibited by SV40 large T-antigen and by HPV-16 E6 oncoprotein. *EMBO J.* **11**:5013–5020.
 31. Miller, A. D. 1992. Retroviral vectors. *Curr. Top. Microbiol. Immunol.* **158**:1–24.
 32. Miller, A. D., and C. Buttimore. 1986. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol. Cell. Biol.* **6**:2895–2902.
 33. Miller, A. D., J. V. Garcia, N. von Suhr, C. M. Lynch, C. Wilson, and M. V. Eiden. 1991. Construction and properties of retrovirus packaging cells based on gibbon ape leukemia virus. *J. Virol.* **65**:2220–2224.
 34. Miller, A. D., and G. J. Rosman. 1989. Improved retroviral vectors for gene transfer and expression. *BioTechniques* **7**:980–990.
 35. Noda, A., S. F. Venable, O. M. Pereira-Smith, and J. R. Smith. 1994. Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression system. *Exp. Cell Res.* **211**:90–98.
 36. Raycroft, L., H. Wu, and G. Lozano. 1990. Transcriptional activation by wild-type but not transforming mutants of the p53 anti-oncogene. *Science* **249**:1049–1051.
 37. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 38. Scheffner, M., J. M. Huibregtse, R. D. Vierstra, and P. M. Howley. 1993. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell* **75**:495–505.
 39. Scheffner, M., K. Munger, J. M. Huibregtse, and P. M. Howley. 1992. Targeted degradation of the retinoblastoma protein by human papillomavirus E7-E6 fusion proteins. *EMBO J.* **11**:2425–2431.
 40. Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine, and P. M. Howley. 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* **63**:1129–1136.
 41. Smotkin, D., and F. O. Wettstein. 1986. Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. *Proc. Natl. Acad. Sci. USA* **83**:4680–4684.
 42. Srivastava, S., Z. Zou, K. Pirollo, W. Blattner, and E. H. Chang. 1990. Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. *Nature* (London) **348**:747–749.
 43. Trask, D. K., and M. T. Muller. 1988. Stabilization of type I topoisomerase-DNA covalent complexes by actinomycin D. *Proc. Natl. Acad. Sci. USA* **85**:1417–1421.
 44. Vogelstein, B. 1990. A deadly inheritance. *Nature* (London) **348**:681–682.
 45. Wassermann, K., J. Markovits, C. Jaxel, G. Capranico, K. W. Kohn, and Y. Pommier. 1990. Effects of morpholinyl doxorubicins, doxorubicin, and actinomycin D on mammalian DNA topoisomerases I and II. *Mol. Pharmacol.* **38**:38–45.
 46. Werness, B. A., A. J. Levine, and P. M. Howley. 1990. Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science* **248**:76–79.
 47. White, R. A., N. H. A. Terry, M. L. Meistrich, and D. P. Calkins. 1990. Improved method for computing potential doubling time from flow cytometric data. *Cytometry* **11**:314–317.
 48. Yin, Y., M. A. Tainsky, F. Z. Bischoff, L. C. Strong, and G. M. Wahl. 1992. Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. *Cell* **70**:937–948.
 49. zur Hausen, H. 1991. Human papillomaviruses in the pathogenesis of anogenital cancer. *Virology* **184**:9–13.