Natural Variants of the Human Papillomavirus Type 16 E6 Protein Differ in Their Abilities To Alter Keratinocyte Differentiation and To Induce p53 Degradation

MELISSA CONRAD STÖPPLER,¹ KAREN CHING,² HUBERT STÖPPLER,¹ KELLY CLANCY,²
RICHARD SCHLEGEL,¹ AND JOSEPH ICENOGLE^{2*}

*Department of Pathology, Georgetown University School of Medicine, Washington, D.C. 20007,¹ and
Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases,
Centers for Disease Control and Prevention, Atlanta, Georgia 30333²*

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Three naturally occurring variant human papillomavirus type 16 (HPV-16) E6 proteins, which contained amino acid substitutions predominantly near the N terminus, exhibited significant differences in their abilities to abrogate keratinocyte differentiation in response to serum and calcium and to induce the degradation of p53 in vitro. One variant surpassed the reference E6 protein in its ability to abrogate keratinocyte differentiation responses, whereas another showed a reduction in this activity. Interestingly, the biological activities of the HPV-16 E6 proteins and their abilities to induce p53 degradation in vitro were directly correlated. These results demonstrate that naturally occurring variants of HPV-16 differ in biological and biochemical properties which might result in differences in pathogenicity.

Human papillomaviruses (HPVs) are a heterogeneous group of viruses associated with cervical cancer as well as benign and premalignant lesions of the cervix (5, 12, 17, 53). These viruses are classified into genotypes on the basis of the nucleic acid sequences of their genomes; individual genotypes of HPV are defined as having L1, E6, and E7 open reading frames (ORFs) that differ by more than 10% (11). Tissue tropism, disease associations, and immortalizing-transforming activities parallel genotype designations. HPV type 6 (HPV-6) and HPV-11 are found primarily in benign exophytic genital lesions, while HPV-16 or HPV-18 are seen in the majority of cervical carcinomas in the United States. The E6 and E7 genes are recognized as the immortalizing-transforming genes of HPVs and have biological activity in both keratinocytes (2, 20, 25) and fibroblasts (4, 8, 31, 52). The E6 and E7 proteins of HPV-16 cooperate to immortalize primary human genital keratinocytes efficiently (41); the HPV-6 E6 and E7 proteins are much less active (19).

Many naturally occurring HPV intratype sequence variants have been identified (6, 10, 14, 16, 21–23, 29, 30, 43, 51). Naturally occurring sequence variants have been found in the upstream regulatory region and the E6, E7, E2, E5, L2, and L1 ORFs (14, 16, 29, 30). Although the biochemical properties of the E6 and E7 proteins from high- and low-risk HPV types vary in a manner consistent with disease associations (19), little is known about intratypic variation in the biological and biochemical properties of E6 and E7 proteins.

The primary focus of this study was to compare the functional properties of variant HPV-16 E6 proteins with those of the reference HPV-16 E6 protein. The reference HPV-16 E6 protein has immortalizing activity when assayed on primary human mammary cells (1). Both immortalization (50) and full transformation (38) of baby rodent kidney cells in cooperation

with an activated *ras* oncogene have been reported for this protein, and it can induce anchorage-independent growth of NIH 3T3 cells (47). The reference HPV-16 E6 protein, but not the reference HPV-6 E6 protein, inactivates the tumor suppressor protein p53 by stimulating its ubiquitination (45). This ubiquitination reaction involves another protein, designated E6-AP, which serves as a ubiquitin donor (26–28, 44). Various studies have shown that E6-stimulated p53 degradation interferes with many known functions of p53, including transcriptional activities (24, 36, 39), G₁ growth arrest for repair of damaged DNA (15, 32), and DNA binding (35).

Sequencing of the E6 and E7 ORFs of HPV-16 DNAs obtained from cervical samples from patients in Spain, Colombia, and Zaire (34, 42) resulted in DNA sequences coding for numerous variant amino acids, which primarily occurred in the E6 protein near its amino terminus. Some amino acid substitutions in this region of the E6 protein are known to alter its activity for degradation of p53 (9, 15). These observations suggested the possibility that these HPV-16 variants have different biological or biochemical properties. We cloned the E6 and E6-E7 ORFs from three representative HPV-16 variants with unique amino acid sequences and the reference HPV-16 DNA and assayed both their abilities to abrogate keratinocyte differentiation in response to serum and calcium (46) and their abilities to induce the degradation of p53. Two of the three variant E6-E7 plasmids expressed proteins which interfered with keratinocyte responses to serum and calcium with an efficiency equal to or greater than that observed for reference E6 and E7 proteins; one variant showed reduced activity. A direct correlation was observed between the biological activities of the variant E6 proteins and their abilities to induce p53 degradation in vitro. These results suggest that natural variants of HPV-16 may have differences in pathogenicity.

MATERIALS AND METHODS

Identification and sequencing of HPV-16 variants. Cervical cells were obtained from women with cervical cancer residing in Spain and Colombia (42) and from a woman enrolled in a human immunodeficiency virus-HPV study in Kinshasa, Zaire, who had cervical intraepithelial neoplasia II (34). Each cervical sample was determined to be HPV-16 DNA positive by stringent nucleic acid

* Corresponding author. Mailing address: Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd. NE, Atlanta, GA 30333. Phone: (404) 639-2179. Fax: (404) 639-0049. Electronic mail address: JCI1@CIDDVD1.EM.CDC.GOV.

FIG. 1. SV40 early promoter-driven vector for expression of variant E6 or E6 plus E7 proteins. DNAs containing the E6 ORF or E6 plus E7 ORFs of the reference HPV-16 or a naturally occurring variant were amplified and cloned into the vector pMSI (see Materials and Methods). Plasmids containing the E6 ORFs alone are referred to as pMSI.ref (for the reference HPV-16 E6), pMSI.z84, pMSI.162, and pMSI.512. Plasmids containing the E6 plus E7 ORFs are designated pM2.ref (reference), pM2.z84, pM2.162, pM2.512, and pM2.z84E6/ref E7 (a hybrid construct containing the E6 ORF of variant z84 and a reference HPV-16 E7 ORF).

hybridization. The sequence of a region of each of the HPV-16 DNAs containing the entire E6 and E7 ORFs was determined by previously described protocols (30). Briefly, a double-stranded DNA containing the E6 and E7 ORFs was amplified by PCR using primers which defined a 902-bp DNA extending from nucleotide (nt) 26 to 928; the primers were 5'-TTCATGTATAAACTAAGG GCGT-3' and 5'-CATAAAACCATCCATTACATCCCGT-3' (48). Single-stranded sequencing templates from both DNA strands were prepared and sequenced by using primers which bound about every 250 nt on one or the other template. This method of sequence determination using bulk (i.e., not cloned) templates has been shown to be insensitive to PCR-generated errors.

Plasmid constructions. The E6-E7 ORFs of HPV-16 DNAs were amplified by PCR, using a 5' primer which contained nt 95 to 119 from the reference (48) HPV-16 upstream regulatory region-E6 sequence, with one nucleotide change from C to T at nt 115. This substitution resulted in no change in the amino acid sequence of the protein but introduced a *Bam*HI site for subcloning purposes. The 3' primer contained the nt 841 to 858 region of the reference HPV-16 E7 sequence plus a sequence of the *Xho*I restriction site downstream of the E7 termination codon. A plasmid containing the reference HPV-16 E6 and E7 ORFs as well as similar plasmids containing variants were prepared and sequenced (*Taq* dideoxy terminator cycle sequencing kit; Applied Biosystems). Analysis was carried out with a model 373 DNA sequencer (Applied Biosystems) with associated software according to the manufacturer's protocol.

Either the E6 ORF or the E6 and E7 ORFs as a unit were subcloned from the intermediate vectors used for sequence determination into a derivative of pSG5 (18) which expresses inserts under the control of the simian virus 40 (SV40) early promoter. This vector, pMSI, includes the consensus Kozak sequence (33), the HPV-16 E6 ATG, the coding sequence for the first four amino acids of E6, a *Bam*HI site, and the HPV-16 E6 3' sequences from nt 553 to the termination codon (nt 557) (Fig. 1). Immediately upstream of the HPV-16 E6 termination codon in pMSI is the restriction site *Ecl*136II, which allows the vector to be cleaved with *Ecl*136II and *Bam*HI for subcloning of HPV-16 E6 fragments cleaved with *Bam*HI and *Pvu*II. The insertion of these HPV-16 E6 fragments preserves the reference amino acid sequence. No naturally occurring sequence variants have been observed in the first four or the last E6 amino acid (51). The *Xho*I restriction site present at the 3' end of the polylinker permits removal of intervening vector sequences and subcloning of variant DNAs cleaved with *Bam*HI and *Xho*I containing the remainder of the E6 and E7 genes. pMSI clones containing either the reference or variant HPV-16 E6 ORF were designated pMSI.ref, pMSI.z84, pMSI.162, and pMSI.512, and clones containing both the E6 and E7 ORFs were designated pM2.ref, pM2.z84, pM2.162, and pM2.512.

For construction of the hybrid plasmid pM2.z84E6/refE7, which contains the E6 ORF from the z84 variant and the reference E7 ORF, a 389-bp *Bam*HI-*Pst*AI fragment of the E6 ORF was removed from clone pM2.z84 and inserted into the vector-E7-containing plasmid pM2.ref cleaved with *Pst*AI and *Bam*HI. All E6 amino acid changes in the z84 variant are contained within this portion of the E6 ORF, and insertion of this fragment into the reference HPV-16 vector resulted in the desired construct.

Cells and cell culture. Primary human foreskin keratinocytes (HFK) were grown in keratinocyte serum-free medium supplemented with epidermal growth factor and bovine pituitary extract (KSFM; GIBCO-BRL). COS cells were passaged in Dulbecco's modified Eagle medium (DMEM) plus 10% fetal bovine serum (FBS). Immortalized keratinocytes were passaged in 3+1 medium (3 parts KSFM plus 1 part DMEM-10% FBS).

Quantitative assay for the induction of differentiation-resistant colonies in primary human keratinocytes. The ability of each E6-E7 construct to induce serum- and calcium-resistant colonies in primary HFK was evaluated by a quantitative assay (46). Primary keratinocytes were grown in 10-cm-diameter dishes to 40% confluence in KSFM, transfected with 10 µg of uncut plasmid DNA by using 50 µl of Lipofectin reagent (GIBCO-BRL), and cultured until the cells reached confluency. The cells were then passaged into 162-cm² flasks and cultured until the cells again reached confluency. Cells were then placed in DMEM containing 10% FBS and 1 µg of hydrocortisone per ml and cultured for an additional 2 to 4 weeks. Colonies appear in this assay as monolayers against a background of differentiated, stratified cells. Macroscopic colonies were quantified after staining with 1% methylene blue. Colonies were also verified microscopically for altered morphology. Alternatively, differentiation-resistant colonies were trypsinized for continued propagation.

Calcium phosphate transfection of COS cells. For transfection of E6-expressing constructs into COS cells, a modified calcium phosphate method was used. Briefly, COS cells were plated at 300,000 cells per dish in 10-cm-diameter dishes. Ten micrograms of DNA in 219 µl of water was mixed with 31 µl of 2 M CaCl₂ and added dropwise with mixing to 250 µl of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered saline (pH 7.05). The plates were incubated for 30 min at room temperature, and the mixture was added to the COS cells in 2 ml of DMEM plus 10% FBS and incubated at 37°C. After 16 to 18 h, cells were shocked for 60 s with 15% glycerol in HEPES-buffered saline, washed three times with phosphate-buffered saline (PBS), and incubated in DMEM-10% FBS.

Immunoprecipitation assays for E6 proteins. COS cells were washed with PBS, incubated with cysteine- and methionine-free DMEM for 1.5 to 2 h, and labeled for 4 h with 400 to 450 µCi of Promix (Amersham), a mixture of [³⁵S]methionine (approximately 75%) and cysteine (25%), in 2 ml of DMEM at 37°C. Cells were washed with PBS and extracted in 1 ml of a modified radioimmunoprecipitation assay buffer (20 mM morpholinopropanesulfonic acid [MOPS], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% deoxycholate, 0.1% sodium dodecyl sulfate [SDS] [pH 7.2]) containing protease inhibitors (0.1 mM tosylsulfonyl phenylalanyl chloromethyl ketone [TPCK], 0.1 mM *N*-α-p-tosyl-L-lysine chloromethyl ketone [TLCK], and 0.5 mM phenylmethylsulfonyl fluoride). After 30 s of vigorous mixing, extracts were centrifuged for 2 min in an Eppendorf microcentrifuge. Twenty microliters of anti-E6 polyclonal antiserum (a gift of J. Schiller) was added to the supernatant along with 50 µl of protein A-Sepharose CL-4B beads (Pharmacia). Following 1.5 h of rotation at 4°C Sepharose beads were pelleted, washed three times in radioimmunoprecipitation assay buffer containing protease inhibitors, and resuspended in 80 µl of SDS sample buffer with β-mercaptoethanol. Samples were heated to 100°C for 4 min and electrophoresed on SDS-12% acrylamide gels. Gels were fixed with glacial acetic acid-methanol, treated with Enlightening (NEN), dried, and exposed to Kodak XAR-5 film at -70°C.

p53 binding and degradation assays. The E6 and human p53 proteins were prepared in a reticulocyte lysate transcription and translation system (TNT; Promega). One microgram of E6 ORF-containing plasmid DNA or 1 µg of plasmid containing the human p53 ORF (from A. Levine, Princeton University) was used in each protein synthesis, and the protein products were labeled as necessary with [³⁵S]cysteine.

For binding studies, labeled E6 protein was mixed with various amounts of unlabeled human p53 in cell lysis buffer (0.1 M Tris-HCl [pH 8.0], 0.1 M NaCl, 1% Nonidet P-40). After a 3-h incubation on ice, the mixtures were allowed to react with an anti-p53 antibody (p53 Ab-1; Oncogene Science) and protein A-Sepharose beads overnight at 4°C. After four washes in cell lysis buffer, bound proteins were released by addition of SDS sample buffer and boiling for 2 min. The samples were then subjected to electrophoresis on SDS-15% polyacrylamide gels. Gels were fixed, dried, and exposed to X-ray film.

For p53 degradation studies, the variant E6 proteins and human p53 were expressed *in vitro* as described above except that all protein products were labeled with [³⁵S]cysteine. The E6 proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) to verify protein integrity and concentration. Equal amounts of each E6 protein and a standard amount of human p53 were mixed and assayed for p53 degradation in 40-µl reaction mixtures containing 6 µl of additional rabbit reticulocyte lysate in 25 mM Tris-HCl (pH 7.5)-100 mM NaCl-3 mM dithiothreitol. After removal of an aliquot for the zero time point, samples were incubated at 25°C, and reactions were stopped at various times by the addition of 1 volume of SDS sample buffer and immediate boiling for 5 min. Samples were analyzed by SDS-PAGE, the gels were fixed and dried, and the radioactively labeled proteins were both visualized by exposure to X-ray film and quantitated by scanning on a Molecular Dynamics 400A PhosphorImager with ImageQuant software (Molecular Diagnostics, Sunnyvale, Calif.). When incubated individually, each of the E6 proteins and the p53 protein were shown to be stable under these reaction conditions. To verify that no additional translation occurred during the degradation studies, unlabeled E6 and p53 were used in a

TABLE 1. Variable amino acids in natural HPV-16 variants

Position	Reference	Amino acid in sequence of:		
		Variant ^a		
		512	162	z84
E6				
10	R	— ^b	G	I
14	Q	H	D	D
64	D	—	E	—
78	H	Y	Y	Y
83	L	V	V	—
138	S	C	—	—
E7				
29	N	—	—	S

^a HPV-16 DNA was amplified from HPV DNA-containing cervicovaginal samples or from the reference clone, cloned into an intermediate vector, and sequenced as described in Materials and Methods. Plasmids which coded for E6 and E7 proteins with amino acid sequences identical to those determined from bulk HPV-16 DNA amplified from the lavage samples were selected for further study.

^b—, reference amino acid.

control degradation reaction to which [³⁵S]cysteine had been added; no radioactive products were formed.

RESULTS

Identification of HPV-16 sequence variants. Sequencing of HPV-16 DNAs amplified from patients' samples identified variants coding for amino acid substitutions at a number of residues in the E6 and E7 proteins, including the first 20 amino acids of the E6 protein, a region previously determined to be important for p53 degradation activity (9, 15). We chose reference HPV-16 DNA and three variant DNAs which coded for proteins with amino acid substitutions in the E6 and E7 ORFs, amplified DNAs containing the E6 and E7 ORFs as a unit by the PCR, cloned the amplified products, and sequenced the resulting clones (Table 1). Each variant had three to five amino acid substitutions in comparison with the reference HPV-16 E6 protein (48). Of the three variants examined, two contained reference E7 protein and one variant (z84) contained an E7 protein with a previously observed amino acid change, an asparagine-to-serine change at amino acid position 29 (N29S) (14, 16, 30).

HPV-16 E6 variant proteins are immunoprecipitable by HPV-16 E6-specific antiserum. The HPV-16 E6 protein has historically been difficult to detect. Indeed, it has sometimes been impossible to precipitate the E6 protein even from cell lines expressing high levels of E6 mRNA (49). However, because a high-avidity antiserum to HPV-16 E6 was available (kindly provided by John Schiller), the immunoprecipitability of the variant E6 proteins was tested. The E6 ORFs of the three HPV-16 sequence variants and the E6 gene from the reference HPV-16 were cloned into the SV40 early promoter-driven vector (Fig. 1) and transiently transfected into COS cells. The COS cell system was chosen to determine if the variant proteins were immunoprecipitable by an HPV-16 E6-specific antiserum because of this cell line's ability to overexpress proteins from SV40 promoter-driven vectors. Figure 2 demonstrates that the E6 variant constructs as well as the reference E6 construct expressed immunoprecipitable E6 protein with an approximate molecular mass of 18 kDa, demonstrating that the constructs expressed stable full-length E6 protein in a conformation which reacts with the HPV-16 E6-specific antiserum.

Natural variants of HPV-16 E6-E7 differ in ability to induce differentiation-resistant colonies in primary HFK. To determine the ability of each of the HPV-16 variants to induce differentiation-resistant colonies in primary HFK, the E6 and E7 ORFs from each variant were first cloned as a unit into pMSI. Seven assays were then done with different donors of HFK for each experiment (Table 2). No donor was used more than once. As expected for different HFK preparations, the number of colonies obtained with the control plasmid pM2.ref varied with HFK preparation.

The HPV-16 variants had significant differences in their abilities to induce differentiation-resistant colonies in primary HFK. In particular, variant 512 consistently led to higher numbers of serum- and calcium-resistant colonies in HFK than reference HPV-16, while variant z84 was reduced in this activity. Variant 162 formed colonies with an efficiency comparable to that seen with reference HPV-16. The average activities for variants z84 and 512 differed by more than four times the larger standard deviation of the results for the individual variants (0.27). Some HFK colonies obtained for each E6 or E7 construct were verified to be immortalized by continued passaging of pooled colonies from each variant in 3+1 medium.

The z84 E6 protein shows reduced ability to cooperate with the reference E7 protein in the formation of differentiation-resistant colonies in primary HFK. Variant z84 was the only variant in the study which contained an amino acid change in the E7 ORF (N29S). To determine whether the z84 E6 protein alone had biological activity different from that of the reference E6 protein, we generated a hybrid plasmid containing the E6 ORF of z84 and the reference E7 ORF (z84E6/refE7). When the activity of this hybrid construct was compared with that of the construct containing the reference E6-E7 ORFs, z84E6/refE7 showed a relative colony formation activity of 0.36 (average of two experiments). Thus, the E6 protein of variant z84 is a less effective enhancer of E7 activity than the reference E6 protein when coexpressed with the reference E7 protein.

The HPV-16 E6 variants have different abilities to target p53 for degradation. A reticulocyte lysate system was used to express the variant E6 proteins and human p53 separately for use in binding and degradation studies. Variant E6 proteins could be immunoprecipitated from mixtures of reticulocyte expressed E6 and p53 by using an anti-p53 monoclonal antibody (Fig. 3). The interaction between HPV-16 E6 proteins and the p53 protein is complex, involving E6, p53, and E6-AP.

FIG. 2. HPV-16 E6 protein expression in COS cells. The E6 ORFs of reference HPV-16 (pMSI.ref) and E6 variants (pMSI.z84, pMSI.162, and pMSI.512) were transfected into COS cells by a modified calcium phosphate precipitation protocol (see Materials and Methods). After 48 h, the cells were radiolabeled with [³⁵S]cysteine-methionine and the E6 proteins were immunoprecipitated. All of the E6 variant constructs as well as the reference E6 construct expressed immunoprecipitable E6 protein with an approximate molecular mass of 18 kDa (arrow). It is likely that the differences in intensity of the E6 protein on the autoradiograph are the result of differences in metabolic labeling and/or immunoprecipitation. Note that the background bands display the same differences in intensity. Positions of molecular mass standards (in kilodaltons) are indicated at the left.

TABLE 2. Differentiation-resistant colony formation activities of E6-E7 constructs

Plasmid	No. of colonies ^a							Avg relative activity ± SD ^c
	Expt 1 ^b	Expt 2	Expt 3	Expt 4	Expt 5	Expt 6	Expt 7	
pMSI	0	0	0	0	0	0	0	0
pM2.z84	10	7	10			12	28	0.56 ± 0.18
pM2.162	18	21	11			18		0.89 ± 0.13
pM2.ref	18	28	14	38	46	18	46	1.0
pM2.512				52	87	31		1.66 ± 0.27

^a The activity of each variant was determined as described in Materials and Methods.

^b Two different DNA preparations were used for experiments 1 to 5 and experiments 6 and 7 to control for any differences in the DNA preparations.

^c The activity of each construct was calculated relative to that of pM2.ref from each experiment (e.g., for experiment 2, the relative activity of pM2.z84 is 0.25), and then the relative activities for all experiments were averaged.

Many HPV-16 E6 proteins mutated by site-directed mutagenesis fail to form E6-p53-(E6-AP) complexes and therefore do not degrade p53 in vitro (49). However, both benign (low-risk) and malignant (high-risk)-associated HPV E6 proteins can bind p53 (37), indicating that p53 binding is not an adequate predictor of HPV biological activity. Thus, to further pursue correlations between biological and biochemical activities of the variant E6 proteins, we analyzed E6-mediated degradation of p53. A standard amount of radiolabeled p53 from a single transcription-translation reaction was mixed with a standard amount of each radiolabeled variant E6 protein, and the degradation of the p53 protein by each E6 protein was monitored by SDS-PAGE and autoradiography (Fig. 4). These autoradiograms showed that the z84 E6 protein had reduced ability relative to the reference E6, 162 E6, or 512 E6 protein to target p53 for degradation.

The quantitative p53 degradation activities of the E6 proteins were determined by phosphor image analysis of the gel shown in Fig. 4. The amounts of radioactivity in both p53 bands and in the E6 band in each lane at the indicated times were determined with a Molecular Dynamics 400A PhosphorImager and associated software. The degradation activity of each E6 protein was calculated by determining the percentage of input p53 remaining at each time (the average of the intensities of all plasmid controls was 1,608 ± 148). At all times, the order for p53 degradation activities was the same (z84 < 162 < reference < 512). For example, at the 1-h time point, 80, 55, 49, and 24% of the input p53 remained for z84, 162, reference and 512, respectively. In addition, the same order of activities was obtained when the phosphor image data were normalized for variation in recoveries of protein in each sample by calculating the changes in the ratio of the amount of p53 protein to the amount of E6 protein at each time point. The order of p53 degradation activities was reproducible in assays using either of two plasmid preparations (data not shown).

FIG. 3. Binding of representative E6 proteins to p53. The indicated E6 variant proteins, labeled with [³⁵S]cysteine, and unlabeled p53 were synthesized separately in vitro. Ten microliters of each of the E6 proteins, 20 μl of cell lysis buffer, and decreasing amounts of p53 protein were mixed (total volume, 40 μl), precipitated with an antibody to p53, and analyzed by SDS-PAGE as described in Materials and Methods. Lanes 1 to 4 contained 10, 5, 2.5, and 1 μl, respectively, of unlabeled p53 protein preparation.

DISCUSSION

The results reported here are the first to demonstrate that naturally occurring variants of the HPV-16 E6 protein differ in at least two important functional activities: the ability to cooperate with the reference E7 protein for the generation of differentiation-resistant HFK colonies and the ability to target the degradation of the human p53 tumor suppressor protein. Furthermore, there was strong correlation between the biological activities of the variants in the keratinocyte assay and their abilities to degrade p53. Variants z84 and 512 had the lowest and highest activities, respectively, for colony formation, and these differences could be attributed to amino acid differences in the E6 proteins. Furthermore, E6 variants z84 and 512 had the lowest and highest activities, respectively, for p53 degradation. The in vitro p53 degradation activities of HPV-16 E6 are known to be due to interactions among HPV-16 E6, E6-AP, and p53 (26–28). However, since E6 has many properties other than p53 degradation, the low and high colony formation activities of variants z84 and 512, respectively, are not necessarily due to altered p53 degradation rates. For example, since HPV-16 E6 and E6BP, a calcium-binding protein, can form complexes (7), the possibility that the variable biological activities are due to changes in HPV-16 E6 activities in these complexes cannot be ruled out. Nevertheless, the consistency in the differentiation-resistant colony formation and p53 degradation activities reported here argues that changes in both activities are the result of the same alterations in the E6 proteins.

The variants used in this study were selected from a larger group of HPV-16 variants with variable amino acids in the E6 and/or E7 proteins (28a). Since it was feasible to analyze only a limited number of variants in the detail described here, three variants with either a large number of amino acid substitutions or nonconservative amino acid substitutions were chosen. Because of these selection criteria, the variants are not representative of the HPV-16 E6-E7 variants in our collection, which predominantly has variants with amino acid substitutions in E6 identical to those found in U.S. populations (51). Furthermore, the variants chosen contain two amino acid substitutions which have not been previously identified (D64E and S138C). Three of the four HPVs studied here did fall into HPV-16 variant classes which have been previously defined by specific amino acid substitutions in the E6 protein (European, Asian, Asian-American, African 1, and African 2) (21, 51). According to these specific amino acid substitutions, variant 512 is an Asian-American variant, z84 is an African 2 variant, and the reference HPV-16 is a European variant. The 162 variant did not fall easily into a defined variant class but had four previously identified variable amino acids (G-10, D-14, Y-78, and V-83). Variants such as variant 162, which lie outside the defined

FIG. 4. Degradation of p53 in the presence of variant E6 proteins. [^{35}S]cysteine-labeled p53 and variant E6 proteins were synthesized separately *in vitro* and then mixed. Samples were removed at 0, 1, 3, and 6 h, boiled in SDS sample buffer, and electrophoresed on SDS-PAGE gels as described in Materials and Methods. A standard volume of a single preparation of p53 was used in each of the degradation reactions; the variant E6 proteins were prepared in parallel to ensure the same incorporation of radioactive cysteine per microgram of E6 protein. No differences in the stability of the variant E6 proteins are apparent; no differences in stability were found by comparing phosphor image data from the bands of variant E6 proteins.

classes of variants when E6 amino acid substitutions alone are considered, have been observed in other studies (51). In summary, the variants chosen for study contained 6 of 11 previously identified variable E6 amino acids and 2 new variable amino acids.

The data described here allowed the identification of specific amino acid residues which may be responsible for the observed quantitative differences in HPV-16 E6 activities. Using the 162 variant of the E6 protein, which has activities very close to those of the reference E6 protein, as the control, it is reasonable to suggest that the properties of the z84 variant of the E6 protein are a result of the R10I substitution, and those of the 512 variant of the E6 protein are the result of the Q14H and/or S138C substitution(s). However, it must be noted that it may be incorrect to assume that differences in the properties of variant E6 proteins are determined by one or two variable amino acids, since these properties may be determined by specific combinations of variable amino acids.

Mutants of the HPV-16 E6 protein which have amino acid changes near the naturally occurring changes studied here have been prepared by site-directed mutagenesis. HPV-16 E6 proteins with substitutions at amino acids 8, 9, and 10 or deletions of amino acids 2 through 12, 9 through 13, 73 through 77, 78 through 82, or 136 through 151 all have low abilities to degrade p53 (9, 15, 49). Furthermore, when six amino acids in the reference HPV-16 E6 protein between positions 106 and 113 are changed to those frequently found in this region in low-risk HPV types, the ability of this protein to bind and degrade p53 is disrupted (49). One conclusion from studies with site-directed mutants is that subtle changes in the E6 protein have major effects on p53 binding and degradation (49). However, all variants described in this report are active compared with the HPV-16 E6 mutants in the same regions constructed by site-directed mutagenesis. Thus, the HPV-16 E6 protein can accommodate some nonconservative amino acid substitutions (e.g., R10I) without large changes in biological and biochemical activities.

The question of whether variant E6 and E7 proteins become established as a unit or as individual proteins remains to be determined. Although there is no evidence that the E6 and E7 proteins interact with each other in a manner which might limit variability in one or both proteins, they do both interact with proteins involved in cell cycle control (p53 and Rb, respectively). It is possible that some fairly narrow range of immortalization activity is optimal for variant establishment and that, over the long evolutionary history of HPV-16 DNAs, variants become established only if the combined immortalization activity of the E6 and E7 proteins is in this narrow range. Since

the HPV-16 E7 N29S variant has been reported to have about a twofold-greater ability to cooperate with the Ras protein in transformation of rat embryo fibroblasts, it was of interest that the N29S HPV-16 E7 variant was found in combination with the z84 E6 and that the combination had lower immortalization activity than the reference E6-E7 (16).

There are at least two factors which would affect the importance of sequence variation on disease prevalence in a population: the prevalence of each variant in that population and any differences in pathogenicity of the variants. The percentages of HPV-16 viruses with reference E7 proteins observed vary from 100% in Germany to 56% in Japan, and the N29S HPV-16 E7 variant protein accounts for about 35% of the E7 proteins found in Japan and Tanzania (14, 16, 30). Other variants of the HPV-16 E7 protein have occasionally been found (14, 16). Many variants of HPV-16 E6 proteins have been observed (51), and the distribution of HPV-16 E6 proteins in various populations is likely to be correspondingly complex. Since at least two major variants of the HPV-16 E7 protein (reference and N29S) and many variants of the HPV-16 E6 protein exist worldwide, the question of whether measurable differences in the pathogenicity of variant viruses exist needs to be answered. Interestingly, the E6-E7 variant in the present study having a reduced activity in the keratinocyte differentiation resistance assay, z84, was isolated from a patient with cervical intraepithelial neoplasia, while the variants with greater activities were each derived from carcinomas.

Naturally occurring variants may display biological differences other than those described here which might affect their pathogenicity. For example, variant-specific differences might occur in host immune response as a result of variation in the 20 N-terminal amino acids of E6. Data supporting the involvement of these amino acids in the human immune response to papillomavirus infections have been reported (3, 13, 40). In addition, recent evidence indicates that the T-cell response to this region of the reference E6 protein is strongly associated with clearing of HPV infection (30a). If selection for escape from immune surveillance drives the establishment of E6 variants, the variation in differentiation-resistant colony formation and p53 degradation activities reported here might be a consequence of covariation of these activities with immune selection.

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