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Mutational Analysis of Human Papillomavirus Type 16 E6 Demonstrates that p53 Degradation Is Necessary for Immortalization of Mammary Epithelial Cells

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We have previously demonstrated that normal human mammary epithelial cells (MECs) are efficiently immortalized by human papillomavirus type 16 (HPV16) E6. HPV16 E6 binds to and induces p53 degradation in vitro and induces a marked reduction of p53 protein in MECs. Low-risk HPV6 E6 is defective for p53 binding and degradation in vitro but immortalized MECs at low efficiency. The HPV6 E6-immortalized MECs had markedly reduced levels of p53. To directly investigate whether the ability of HPV16 E6 to stimulate p53 degradation is required for E6-induced immortalization, a series of HPV16 E6 mutants were analyzed for the ability to bind and degrade p53 in vitro, induce a reduction in p53 levels in vivo, and immortalize MECs. We observed that one set of mutants efficiently immortalized MECs, caused a reduction in p53 levels in vivo, and degraded p53 in vitro. Other mutants immortalized MECs with low efficiency and either induced p53 degradation at low levels or were unable to induce p53 degradation in vitro; however, all of the immortal clones displayed low levels of p53. A third class of mutants did not immortalize MECs and failed to induce a reduction in p53 levels in vivo or degrade p53 in vitro. These results demonstrate that a reduction in p53 protein levels due to enhanced degradation is essential for MEC immortalization by HPV16 E6.

Epithelial cancers account for the vast majority of human tumors. An understanding of the biochemical basis of early oncogenesis has been elusive because of the lack of efficient in vitro human cell transformation models. Most oncogenic agents that transform rodent cells do not transform human cells efficiently. The introduction of human papillomavirus (HPV) DNA into cultured human epithelial cell types and smooth muscle cells results in their immortalization (5–7, 9, 15, 16, 24, 25, 32). While human keratinocytes required both the E6 and E7 oncogenes for efficient immortalization (6, 7, 9, 15, 16, 24, 32), we have demonstrated that one subtype of primary human mammary epithelial cells (MECs) is uniquely immortalized by a single oncogene, HPV type 16 (HPV16) E6 (2, 3, 36).

The molecular events underlying the HPV16 E6-induced immortalization of MECs are not known. One of the biochemical functions of HPV16 E6 is to interact with the p53 tumor suppressor gene product. The E6 proteins of the high-risk viruses complex with p53 in vitro (38), resulting in selective degradation of p53 via the ubiquitin pathway (11, 31). Consistent with these in vitro results, HPV16 E6-immortalized human MECs showed nearly undetectable levels of p53 protein, whereas the parental cells expressed relatively high levels of p53 protein (2, 3, 13). The HPV16 E6-induced p53 loss was due to enhanced degradation, reflected in a sixfold reduction in the half-life of the p53 protein (2). The MEC system allowed us to compare and contrast the properties of the high-risk E6 proteins with low-risk HPV6 E6 and bovine papillomavirus type 1 (BPV1) E6. MECs transfected with these genes underwent a crisis period with extensive cell death and the subsequent emergence of immortal cells. While the pre-crisis cells had significant levels of p53 protein, the established HPV6 or BPV1 E6-immortalized MECs had low p53 levels due to a reduction in the half-life of p53, similar to HPV16 E6-immortalized MECs (2). While HPV6 E6 does not mediate p53 degradation in vitro, two groups have reported discrepant results on the ability of low-risk E6 to bind p53. Scheffner et al. (31) were unable to detect complex formation between HPV6 and HPV11 E6 with p53 in vitro. In contrast Crook et al. (11) detected low-efficiency association between HPV6 and HPV11 E6 and p53 in vitro, with no degradation of the complexed p53. BPV1 E6 does not bind to or mediate p53 degradation in vitro (12, 38).

Recent analyses have revealed that E6 proteins from the high-risk viruses bind a polypeptide, E6-AP, present in rabbit reticulocyte lysate, and this association is required for both p53 binding and degradation in vitro (18). The E6/E6-AP complex functions as a ubiquitin ligase in the degradation of p53 (29) and interacts with a specific ubiquitin-conjugating enzyme (28). E6-AP did not bind E6 proteins from low-risk viruses such as HPV6 and HPV11 in vitro (19). However, an HPV6 E6 hybrid containing the E7-Rb binding domain targeted the complexed Rb protein for degradation in vitro (30), and E6-AP is functionally required for this interaction. Furthermore, BPV1 E6 can bind to E6-AP at levels similar to those for HPV16 E6 (10). These results demonstrate that the E6 proteins from the low-risk viruses interact with E6-AP.

To directly address whether the ability of HPV16 E6 to target p53 for degradation is necessary for MEC immortalization, we tested a panel of random substitution mutants of HPV16 E6 for the ability to immortalize human MECs and to induce p53 degradation in vitro and in vivo. These analyses demonstrate that p53 degradation is essential for the immortalization of MECs by HPV16 E6. Furthermore, a subset of mutant E6 genes show an intermediate immortalization phenotype, similar to the one exhibited by HPV6 and BPV1 E6.

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These mutants should serve as valuable tools with which to further dissect the nature of differential immortalization by high- and low-risk papillomavirus E6 genes.

MATERIALS AND METHODS

HPV16 E6 constructs and mutagenesis. HPV16 E6 was PCR amplified as two fragments, using the oligonucleotide primers 5′ gcg gtc gac ATG CAC CAA AAG AGA AC 3′, 5′ TCT TCC GGA CAC AGT GGC 3′, 5′ GTG TCC GGA AGA AAA GC 3′, and 5′ cgc gaa gcT TAC AGC TGG GTT TC 3′ (linker sequences are in the lowercase, and coding sequences are in uppercase), and cloned in a three-way ligation as a *Sal*I-*Hin*dIII fragment into pUC19. Wild-type HPV16 E6, Glu-113 Stop, Cys-111 Tyr/Gln-116 His/Arg-117 Lys/Arg-124 Stop, and Arg-124 Gly were PCR amplified as an *Eco*RI-*Bam*HI cassette, using the primers 5′ gcg aat tcg tcg acc ATG CAC CAA AAG AGA AC 3′ and 5′ cgc gga tcc aag cTT ACA GCT GGG TTT C 3′, and cloned into the vector pLXSN (23) to generate recombinant retroviruses to infect MECs. Δ73-77 and Δ118-122 (provided by K. Vousden and T. Crook) were cloned as an *Eco*RI-*Hin*cII fragment into the vector pLXSN. All the other mutants were cloned as a *Sal*I-*Bsp*EI cassette into the wild-type HPV16 E6 gene in pLXSN. The mutant E6 genes were cloned into pSP65 (Promega) as an *Eco*RI-*Bam*HI fragment. p16E6SP65 and pProp53SP65 were obtained from K. Vousden. All constructs were sequenced to confirm the wild-type or mutant sequences. The HPV16 E6 gene was subjected to random mutagenesis, using a PCR-based misincorporation protocol (20). The gene was mutagenized in three blocks, using the following pairs of oligonucleotides: 5′ gcg gtc gac ATG CAC CAA AAG AGA AC 3′ and ACT ATG CAT AAA TCC CG 3′; 5′ TTT ATG CAT AGT ATA TAG 3′ and 5′ TCT TCC GGA CAC AGT GGC 3′; and 5′ GTG TCC GGA AGA AAA GC 3′ and 5′ cgc gaa gcT TAC AGC TGG GTT TC 3′. The products of three independent reactions were separately inserted into the wild-type HPV16 E6 gene in pUC19 and transformed into *Escherichia coli*. Individual colonies were selected from each population, and the mutagenized region was sequenced by using Sequenase version 2.0 (United States Biochemical) and the M13 sequencing primers (New England Biolabs) to screen for mutant clones. Cys-63 Ser, Cys-63 Arg, and Cys-106 Arg were created by site-directed mutagenesis, using the oligonucleotides 5′ ggg CAT ATG CTG TA(T/G) GTG 3′ and 5′ cgg TCC GGA CAC AGT TTC TTT TGA C(C/G/T)G T 3′. These oligonucleotides were used with a wild-type HPV16 E6 oligonucleotide to amplify a DNA fragment that contained the mutation. These fragments were subcloned into wild-type HPV16 E6 in pSP65, and the presence of the mutation was confirmed by DNA sequencing. These mutant E6 genes were cloned into pLXSN as an *Eco*RI-*Bam*HI fragment.

Retrovirus packaging. The pLXSN-based constructs were introduced into the amphotropic packaging cell line PA317 (obtained from D. Galloway and A. D. Miller), using calcium phosphate precipitation (23). The transfectants were selected in 1 mg of G418 per ml. Colonies of cells that produced high-titer viruses on the 76R-30 (radiation-transformed 76N MECs) cell line (35) were switched to DFCI-1 medium (4) for 16 h prior to collection of viral supernatants, and approximately equal numbers of CFU were used to infect MECs.

Immortalization assays. The normal mammary epithelial cell strain 76N was derived from a reduction mammoplasty specimen and grown in DFCI-1 medium as described earlier (4). 76N cells (10^5 to 10^6/100-mm-diameter dish) in DFCI-1 medium were infected with approximately equal numbers of CFU of the retrovirus supernatants from PA317 producer cell lines containing either vector, wild-type HPV16 E6, or various E6 mutants as described by Halbert et al. (15). For the initial experiments, G418 (50 μg/ml) selection was started 48 h after infection and continued for 10 days. At that time, G418-resistant cells were pooled and either maintained in G418-containing DFCI-1 medium or shifted to D2 medium (DFCI-1 medium lacking fetal calf serum and bovine pituitary extract but supplemented with 0.05% bovine serum albumin), which allows growth of immortal but not normal mammary epithelial cells (2, 3, 5). At least three independent experiments were performed with each mutant. Emergence of immortal cells without any crisis period (similar to wild-type HPV16 E6) is scored as efficient immortalization. However, if the infected cells exhibited a crisis period and did not give rise to immortal clones in all the experiments (similar to HPV6 E6), they were scored as inefficient for immortalization.

In vitro binding and degradation assays. In vitro transcription using SP6 RNA polymerase, in vitro translation in rabbit reticulocyte lysates (Promega) in the presence of [^{35}S]cysteine, and binding/degradation reactions were performed as described previously (11) except that 110 ng of baculovirus-derived p53 (a generous gift of Stephen Fawell, Biogen Inc.) instead of reticulocyte lysate-synthesized p53 protein was used per binding reaction. p53 protein was incubated with equal amounts of in vitro-translated ^{35}S-labeled E6 proteins together with the p53-specific monoclonal antibody (MAb) pAb421 and protein A-Sepharose beads in low-salt association buffer (100 mM NaCl, 100 mM Tris-HCl [pH 8.0], 0.1% Nonidet P-40 for 1 h at 4°C. The beads were washed four times with low-salt association buffer and subjected to electrophoresis on a sodium dodecyl sulfate (SDS)–15% polyacrylamide gel. All solutions contained the protease inhibitor phenylmethylsulfonyl fluoride at 1 mM. For degradation assays, p53 protein was translated in reticulocyte lysate in the presence of [^{35}S]cysteine and [^{35}S]methionine. One microliter of the p53 translation mixture was incubated at 30°C for 3 h in 25 mM Tris (pH 7.5)–100 mM NaCl–3 mM dithiothreitol with equal amounts of the different E6 proteins as determined by SDS-polyacrylamide gel electrophoresis (PAGE). The reaction products were resolved on an SDS–10% polyacrylamide gel and visualized by autoradiography. For the temperature-sensitive assays, the reactions were performed at 25, 30, and 37°C.

Immunoprecipitation of p53 and E6 proteins. 76N cells (10^5 to 10^6/100-mm-diameter dish) in DFCI-1 medium were infected with approximately equal numbers of CFU of the retrovirus supernatants containing either wild-type HPV16 E6 or various E6 mutants. After 48 h, cells were labeled with [^{35}S]methionine and [^{35}S]cysteine in cysteine- and methionine-free medium, immunoprecipitated, and analyzed for p53 protein as described previously (2). To assess the expression of the nonimmortalizing E6 mutants, an isogenic derivative of 76N cell strain, 76R-30 (35), was infected with recombinant E6 retroviruses, selected in G418 for 10 days, and then labeled with [^{35}S]cysteine. The immunoprecipitations were performed as described earlier (1), and the gels were analyzed on a Bio-Rad Molecular Imager.

RESULTS

p53 binding and degradation in vitro by HPV16 E6 mutants. To examine the requirements for E6 immortalization of MECs, we generated a series of substitution mutants in HPV16 E6 by using a PCR-based misincorporation technique and site-directed mutagenesis. Mutants with missense or nonsense substitutions, as determined by DNA sequencing, were subcloned into pSP65 to synthesize RNA for in vitro translation or into the retroviral expression vector pLXSN for retrovirus production. As a first step, the abilities of mutant E6 proteins to bind and degrade p53 in vitro were measured. In vitro-translated ^{35}S-labeled E6 proteins were incubated with p53 protein purified from insect cells and immunoprecipitated with anti-p53 MAb pAb421, and the bound E6 proteins were analyzed by SDS-PAGE. To assess p53 degradation, in vitro-translated ^{35}S-labeled p53 protein was incubated with equal amounts of in vitro-translated E6 protein, and the reaction products were analyzed by SDS-PAGE. As expected, wild-type HPV16 E6 bound (Fig. 1A, lane 1) and stimulated degradation of p53 in vitro (Fig. 1B, lane 2). Seven of the twenty mutants bound and degraded p53 in vitro as efficiently as wild-type HPV16 E6 (Table 1); results for six of these mutants are shown in Fig. 1A (lanes 5, 7, 11, 13, 15, and 17). Five mutants, such as Tyr-84 Cys (Table 1) and Arg-124 Gly (lane 25), displayed reduced p53 binding. Tyr-84 Cys bound p53 at low efficiency yet mediated p53 degradation as efficiently as wild-type HPV16 E6 (Table 1). In contrast, Arg-124 Gly showed reduced p53 degradation in vitro (Fig. 1B, lane 14). Eight mutants which did not bind p53 (Fig. 1A, lanes 3, 9, 19, 21, and 23) also failed to induce p53 degradation in vitro (Fig. 1B, lanes 3, 6, 11, 12, and 13). These mutants provided reagents with which to analyze the relationship between the ability of HPV16 E6 to bind and degrade p53 in vitro and to immortalize and reduce p53 protein levels in MECs.

Loss of p53 protein induced by HPV16 E6 mutants at early passages. MECs infected with the different E6 retroviruses were assayed for p53 levels both at early passages after infection and at late passage in the cases in which immortal clones were obtained. At early passages, p53 protein levels were nearly undetectable in cells infected with a virus expressing HPV16 E6 (Fig. 2, lane 2; Table 1). One class of mutants induced a reduction in p53 levels in vivo comparable to that found for wild-type HPV16 E6 (Table 1). A representative example of this phenotype was Tyr-84 Cys (Fig. 2, lane 6). Arg-124 Gly also induced a significant decrease in p53 levels in vivo in early-passage MECs, though not at wild-type levels (Fig. 2, lane 8); similar results were observed in vitro (Table 1; Fig. 1B). Cells infected with mutants such as Cys-106 Arg and Cys-111 Tyr/Gln-116 His/Arg-117 Lys/Arg-124 Stop expressed levels of the p53 protein equivalent to those found in normal 76N cells infected with the vector alone (Fig. 2, lanes 4 and 10, respectively; Table 1). All of the mutants that mediated p53

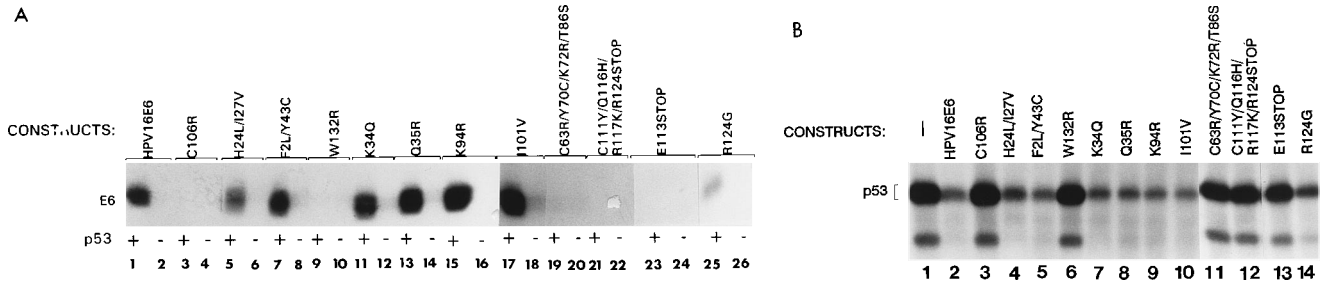

FIG. 1. (A) In vitro binding of HPV16 E6 mutants to human p53. The different E6 proteins were translated in vitro in the presence of [^{35}S]cysteine, and the products were immunoprecipitated with anti-p53 MAb pAb421 in the absence (−) or presence (+) of 110 ng of purified p53 protein. The E6 translations were analyzed by SDS-PAGE to ascertain that equal amounts of E6 proteins were used in all reactions. Wild-type HPV16 E6 bound p53 (lane 1), as did 7 of 12 mutants represented here (lanes 5, 7, 11, 13, 15, 17, and 25). Other mutants were unable to interact with p53 in vitro (lanes 3, 9, 19, 21, and 23). All lanes are from the same experiment. (B) In vitro degradation of p53 by HPV16 E6 mutants. The p53 protein was translated in vitro in the presence of [^{35}S]cysteine and [^{35}S]methionine and incubated with in vitro-translated HPV16 E6 proteins (lanes 2 to 14) or a distilled-water-primed lysate (lane 1) for 3 h. The E6 translations were analyzed by SDS-PAGE to ascertain that equal amounts of E6 proteins were used in all reactions. The reaction mixture was then loaded on an SDS–10% polyacrylamide gel. Note that HPV16 E6 targeted p53 for degradation in vitro (lane 2). Seven of twelve mutants (lanes 4, 5, 7, 8, 9, 10, and 14) also targeted p53 for degradation, while other mutants (lanes 3, 6, 11, 12, and 13) did not target degradation of p53 in vitro. All lanes are from the same experiment.

degradation in vitro also induced a reduction in p53 levels in vivo at early passage, except for the mutants Cys-63 Gly, Cys-63 Ser, and Cys-63 Arg (Table 1).

A subset of HPV16 E6 mutants are temperature sensitive for p53 degradation in vitro. Cys-63 forms the base of the putative first zinc finger. Mutations to Gly, Ser, and Arg induced p53 degradation in vitro but not in vivo at early passage. One possibility was that these E6 proteins are temperature sensitive for the ability to induce p53 degradation. In vitro degradation reactions were performed at 25, 30, and 37°C (Fig. 3). Wild-type E6 induced p53 degradation at all three temperatures tested (lane 2), in contrast to a distilled-water-primed lysate (lane 1) or a mutant, Cys-106 Arg, that does not bind to p53 (lane 6). Interestingly, all three cysteine mutants (Cys-63 Gly, Cys-63 Ser, and Cys-63 Arg) induced p53 degradation at 25 and 30°C (lanes 3 to 5). However, at 37°C, Cys-63 Gly and Cys-63 Ser were completely unable to induce p53 degradation (lanes 3 and 4), and Cys-63 Arg induced p53 degradation at lower levels than wild-type E6 (lane 5). The inability or reduced ability of these mutants to induce p53 degradation at 37°C in vitro correlates with their inability to reduce p53 levels in vivo at early passage after infection of MECs with a retrovirus expressing these mutant genes. We were unable to perform similar in vivo assays, as the cells did not grow adequately at 30°C. None of the other mutants reported here displayed a temperature-sensitive phenotype in vitro (data not shown).

TABLE 1. p53 binding and degradation by HPV16 E6 mutants and p53 levels in MECs[a]

Construct	In vitro p53[b]		p53 level in MECs (at early passage)
	Binding	Degradation	
HPV16 E6	High	++	Low
Phe-2 Leu/Tyr-43 Cys	High	++	Low
His-24 Arg	High	++	Low
His-24 Leu/Ile-27 Val	High	++	Low
Lys-34 Gln	High	++	Low
Gln-35 Arg	High	++	Low
Tyr-84 Cys	Low	++	Low
Lys-94 Arg	High	++	Low
Ile-101 Val	High	++	Low
Cys-63 Ser	Low	+	High
Arg-124 Gly	Low	+	Low
Trp-132 Arg	None	−	High
Cys-63 Gly	Low	+	High
Cys-63 Arg	Low	++	High
Cys-63 Arg/Tyr-70 Cys/Lys-72 Arg/ Thr-86 Ser	None	−	High
Tyr-76 Stop	None	−	High
Lys-94 Stop	None	−	High
Cys-106 Arg	None	−	High
Cys-111 Tyr/Gln-116 His/Arg-117 Lys/Arg-124 Stop	None	−	High
Glu-113 Stop	None	−	High
Asp-120 Gly/Ile-128 Met/Arg-131 Pro	None	−	High

[a] Amino acid substitutions (one to four) in each mutant are indicated. The mutants were tested for the ability to bind and degrade p53 in vitro and the ability to induce p53 degradation in vivo at early passage. "None" indicates no detectable binding. The degradation assays were scanned on a Molecular Dynamics PhosphorImager.

[b] Each point is the average of at least three experiments. ++, 71 to 100% of the level for wild-type HPV16 E6; +, 41 to 70% of the level for wild-type HPV16 E6; −, 0 to 15% of the level for wild-type HPV16 E6. Fifteen percent is within the range of variation that we observed in different experiments. MECs were infected with various mutants and analyzed for p53 protein by immunoprecipitation 48 to 72 h later.

Immortalization of normal MECs by E6 substitution mutants. Cells infected with retroviruses expressing the HPV16

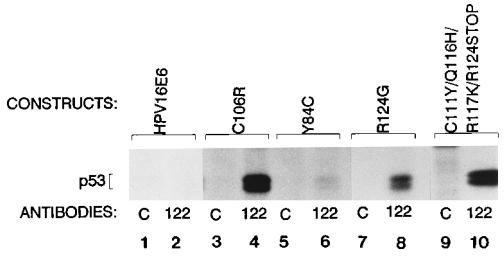

FIG. 2. In vivo loss of p53 induced by HPV16 E6 in MECs. MECs were infected with the recombinant retroviruses, and after 48 to 72 h of infection, NETN lysates of metabolically labeled cells were immunoprecipitated with anti-p53 MAb pAb122 (lanes labeled 122) or P3 (lanes labeled C) as a negative control. Cells infected with virus carrying the HPV16 E6 gene had low levels of p53 (lane 2), as did the mutants that gave rise to immortal clones such as Tyr-84 Cys (lane 6). Cells infected with virus carrying the mutant Arg-124 Gly (lane 8), which immortalized cells after a crisis period, had significantly lower levels of p53 than did mutants that did not give rise to immortal clones. Mutants such as Cys-106 Arg (lane 4) and Cys-111 Tyr/Gln-116 His/Arg-117 Lys/Arg-124 Stop (lane 10) that did not decrease p53 levels in vivo also failed to immortalize MECs.

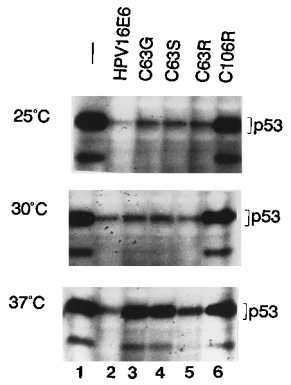

FIG. 3. Temperature-sensitive degradation of p53 by HPV16 E6 mutants. p53 degradation assays were performed at 25, 30, and 37°C, and the reaction products were analyzed on an SDS–10% polyacrylamide gel and visualized by autoradiography. HPV16 E6 induced p53 degradation at all three temperatures (lane 2), compared with a control distilled-water-primed lysate (lane 1) or Cys-106 Arg, which does not bind to p53 (lane 6). Cys-63 Gly, Cys-63 Ser, and Cys-63 Arg induced p53 degradation at 25 and 30°C (lanes 3 to 5). At 37°C, the abilities of these mutants to induce p53 degradation were dramatically reduced (lanes 3 to 5, lower panel).

E6 mutants were transferred to D2 medium to test for the immortal phenotype. In addition to the substitution mutants described above, we also included two previously isolated E6 mutants with five amino acid deletions (obtained from K. Vousden) for these analyses. It had been shown that E6 mutant Δ118-122 induced p53 degradation in keratinocytes and inhibited the p53-mediated G_1 arrest, whereas the mutant Δ73-77 was defective for both properties (14).

Assessment of the immortalizing abilities of these E6 mutants revealed three distinct phenotypes. Nine of the twenty-two mutants tested (Table 2) were able to immortalize MECs as efficiently as wild-type HPV16 E6 in all three independent experiments. Furthermore, like wild-type E6, these mutants yielded immortal cells with a short latency and without passing through a crisis period. All of the immortal clones had markedly reduced levels of p53 protein (Table 2). The second category of mutants, such as Cys-63 Ser, Arg-124 Gly, and Trp-132 Arg, immortalized MECs with a lower efficiency than wild-type E6 (Table 2), a phenotype previously observed with HPV6 and BPV1 E6 (2). One of three or six independent experiments yielded immortal clones with a long (about 1 month) crisis period. In contrast to HPV6 E6, however, Arg-124 Gly induced p53 degradation in vitro and in vivo (Table 1), though at a lower level than HPV16 E6, which may account for the low efficiency of immortalization. However, Cys-63 Ser and Trp-132 Arg were unable to target p53 for degradation in vitro or induce p53 degradation in MECs at early passage (Table 1). Interestingly, the established immortal clones of MECs expressing these mutants had low p53 levels similar to those of HPV16 E6-immortalized cells (Table 2), a phenotype similar to that observed with HPV6 E6 and BPV1 E6 (2). The third category of mutants, such as Cys-63 Gly, Cys-106 Arg, and Δ73-77, were defective for immortalization in this assay in all four independent experiments (Table 2). All of the immortalization-defective mutants were unable to induce p53 degradation in vitro or at early passages in vivo (Table 1). We were unable to assay p53 levels in cells infected with these mutants at late passage since they did not give rise to immortal clones.

Immunoprecipitation of E6 proteins. We assayed for in vivo expression of the different E6 proteins by immunoprecipitation in the E6-immortalized cells as described previously (1). All of the immortal cells expressed E6 protein, as expected, although prolonged exposures of the gels were required (Table 2). To ascertain whether the immortalization-defective E6 mutants expressed stable proteins in vivo, we tried to immunoprecipitate E6 protein from infected cells at early passages. However, these analyses were not successful, as it is difficult to obtain sufficient quantities of actively proliferating cells for labeling and subsequent immunoprecipitation analysis. Therefore, we infected a 76N isogenic cell line, 76R-30 (35), with various recombinant E6 retroviruses and then selected in G418 for 10 days. G418-resistant cells were then labeled with [^{35}S]cysteine, and the immunoprecipitations were performed as described previously (1). As seen in Fig. 4, both immortalization-defective mutants and mutants that immortalized at low efficiency were present at levels comparable to those of wild-type HPV16 E6. Most of the immortalization-defective mutants were detected in this assay (Table 2) except for five mutants, of which four contained stop codons. Our inability to detect these mutants may be due to the fact that they are unstable or are not labeled efficiently because of the truncations.

DISCUSSION

The genital papillomaviruses can be separated into two classes reflecting their association with malignant lesions such

TABLE 2. Immortalization of MECs by HPV16 E6 mutants[a]

Construct	p53 level (in immortalized MECs)	Immortalization[b]	HPV16 E6 protein[c]
HPV16 E6	Low	6 (6)	+
Phe-2 Leu/Tyr-43 Cys	Low	3 (3)	+
His-24 Arg	Low	3 (3)	+
His-24 Leu/Ile-27 Val	Low	3 (3)	+
Lys-34 Gln	Low	3 (3)	+
Gln-35 Arg	Low	3 (3)	+
Tyr-84 Cys	Low	3 (3)	+
Lys-94 Arg	Low	3 (3)	+
Ile-101 Val	Low	3 (3)	+
Δ118-122	Low	3 (3)	+
Cys-63 Ser	Low	1 (3)[d]	+
Arg-124 Gly	Low	1 (6)[d]	+
Trp-132 Arg	Low	1 (3)[d]	+
Cys-63 Gly		0 (4)	+
Cys-63 Arg		0 (4)	+
Cys-63 Arg/Tyr-70 Cys/Lys-72 Arg Thr-86 Ser		0 (4)	+
Δ73-77		0 (4)	+
Tyr-76 Stop		0 (4)	−
Lys-94 Stop		0 (4)	−
Cys-106 Arg		0 (4)	+
Cys-111 Tyr/Gln-116 His/Arg-117 Lys/Arg-124 Stop		0 (4)	−
Glu-113 Stop		0 (4)	−
Asp-120 Gly/Ile-128 Met/Arg-131 Pro		0 (4)	−

[a] Cells (10^5 to 10^6) were infected with retroviruses carrying the different E6 genes. Amino acid substitutions (one to four) in each mutant are indicated. The infected cells were analyzed for p53 protein by immunoprecipitation with pAb122 in the immortal clones and transferred to D2 selection medium to test for the immortal phenotype.

[b] Number of experiments that yielded immortal cells. The number of experiments performed is in parentheses.

[c] In the case of intermediate and defective E6 mutants, 76R-30 cells were used to detect E6 protein as described in Materials and Methods.

[d] The cells went through a crisis period of about 1 month before the emergence of immortal cells.

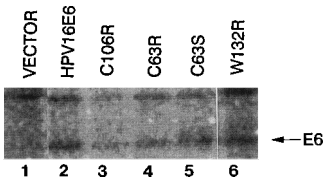

FIG. 4. Expression of the mutant E6 proteins in 76R-30 cells. 76R-30 cells were incubated with recombinant retroviruses carrying the different E6 proteins. After selection for 10 days in G418, the cells were plated at approximately 80% confluence and labeled with [^{35}S]cysteine. Lysates of the labeled cells were immunoprecipitated with the anti-HPV16 E6 antibody 166-2. 76R-30 cells infected with vector showed no E6 protein, as expected (lane 1). Mutants such as Cys-106 Arg, Cys-63 Arg, Cys-63 Ser, and Trp-132 Arg (lanes 3 to 6) showed levels of E6 protein comparable to levels of wild-type HPV16 E6 (lane 2). All the lanes are from the same experiment.

as cervical cancer. HPV16 is the most common of the high-risk HPVs, while low-risk viruses such as HPV6 are rarely isolated in malignant lesions (39). By using in vitro models, it has been demonstrated that HPV16 E6 and E7 genes can induce the immortalization of primary human keratinocytes (6, 7, 9, 15, 16, 24, 32). We have recently shown that mammary tissue contains multiple epithelial cell types which differ in the response to immortalization by the HPV E6 and E7 oncogenes (36). One subtype, predominantly observed in late passages, is uniquely immortalized by HPV16 E6 alone (3, 5, 36). Although HPVs are not associated with breast cancer, immortalization of MECs induced by a single gene provides a unique model with which to understand the molecular basis of immortalization of breast cells as well as the biologic properties of an important viral oncogene.

The mechanisms by which HPV16 E6 transforms cells remain to be determined. One important mechanism may be the ability of HPV16 E6 to bind to and induce p53 degradation in vitro (31). Consistent with this observation, the half-life of p53 is reduced in HPV16 E6-immortalized MECs (2) and in keratinocytes immortalized by E6 and E7 (17). By using high-titer retrovirus to introduce HPV16 E6, the reduction in the half-life of p53 protein was found to be an early event, detected 48 to 72 h postinfection and prior to transfer of the cells to selection medium. We have exploited the MEC model to address whether p53 degradation is a critical function of HPV16 E6 in immortalization. Twenty HPV16 E6 mutants were tested for the ability to interact with p53 and immortalize MECs. Our data strongly suggest that enhanced p53 turnover is required for E6-induced immortalization. Mutants that immortalized MECs and induced low p53 levels in vivo with wild-type efficiency also bound and efficiently induced p53 degradation in vitro. Furthermore, all mutants that induced p53 degradation at 37°C in vitro did so in vivo (Fig. 3 and data not shown). This correlation suggests that the E6-AP-dependent E6-induced p53 degradation observed in vitro occurs in vivo. All nine mutants that failed to immortalize MECs were defective for p53 degradation in vitro and in vivo. We have not isolated any E6 mutants that induce immortalization without a concomitant reduction in the levels of p53 or mutants that induce p53 degradation in vitro and in vivo and are defective for immortalization. These results strongly suggest that p53 degradation is required for immortalization.

Importantly, certain HPV16 E6 mutants exhibited an immortalization phenotype similar to that of HPV6 and BPV1 E6, which do not induce p53 degradation in vitro (2). HPV6 and BPV1 E6-immortalized clones demonstrated a reduced p53 half-life (2) and undetectable protein by Western blot (immunoblot) analysis (12). Two HPV16 E6 mutants which are p53 degradation defective in vitro, Cys-63 Ser and Trp-132 Arg, immortalized MECs with low efficiency. Early-passage cells expressing these mutants contained normal to slightly reduced p53 protein levels, consistent with the in vitro results. The immortal clones all demonstrated rapid p53 turnover, resembling wild-type HPV 16 E6. While the mechanisms by which E6 induces p53 degradation in MECs are not defined, one likely pathway, reflected in the in vitro assay, is through complex formation with E6-AP with the subsequent ubiquitination and proteolysis of p53. It is also possible that HPV16 E6 mediates p53 loss in vivo through another pathway distinct from that in rabbit reticulocyte lysates. For instance, we have demonstrated that a subtype of MECs express markedly lower levels of Rb subsequent to immortalization by HPV16 E7 (36). HPV16 E7 has been shown to complex with Rb but does not induce its degradation in vitro (30). These results suggest that the HPV oncogenes have evolved multiple strategies to inactivate proteins that suppress cell growth. Arg-124 Gly induced p53 degradation at levels lower than those of wild-type E6 both in vitro and in vivo, and this may explain the low efficiency of immortalization by this mutant. Regarding the intermediate class of mutants, it remains possible that a cellular mutation that induces p53 degradation has occurred. However, this is unlikely since spontaneous immortalization has never been observed with these MECs, and this phenomenon is not observed with the defective E6 mutants. The second possibility, that the E6 mutants are undergoing a second-site mutation, is also unlikely, as we have sequenced the entire coding region of the E6 gene from Trp-132 Arg-immortalized cells and found it to have only the expected mutation. In addition, we have previously shown that HPV6 E6, which resembles these mutants in both in vitro and in vivo behavior, lacked any mutations in immortal MECs when the entire coding region was sequenced (2). Taken together, these results imply that p53 degradation is essential for E6-mediated MEC immortalization.

In contrast to our study, Pim et al., using HPV 18 E6 and v-Ras in a mouse kidney cell transformation model, observed that E6 mutants defective for p53 degradation in vitro retained transformation capability (26). This apparent discrepancy with our findings may be due to the different cell types used in the two studies. While our findings demonstrate that p53 degradation is essential for HPV16 E6-induced immortalization, it is possible that other E6 functions are required, but not sufficient, to induce MEC immortalization. For example, using the yeast two-hybrid system, we have identified another E6-binding protein, called E6BP (10). E6BP is identical to ERC55, a putative calcium-binding protein localized to the endoplasmic reticulum (10, 37). The interaction of HPV16 E6 with E6BP/ERC55 may represent a function in E6-induced immortalization or transformation. Studies to test this possibility are in progress.

There is a discrepancy in the literature about the separation of the p53 binding and degradation functions in HPV16 E6 (11, 14). In this study, we were unable to map separate domains in HPV16 E6 for p53 binding and degradation. In the series reported here, mutations that did not alter p53 binding and degradation involved amino acids not found in all genital HPVs (such as His-24 Arg) or represent conservative changes for charge or hydrophobicity in well-conserved amino acids (such as Ile-101 Val). Mutations that abrogated p53 binding represented nonconservative changes in conserved amino acids, such as Cys-106 Arg and Trp-132 Arg. From analysis of temperature-sensitive HPV16 E6 mutants, we suggest that the finger structures are required to mediate p53 binding and degradation in vitro and in vivo. The cysteine residue (Cys-63) affected is at the base of the N-terminal zinc finger and is

presumably required for zinc coordination, while mutations at Cys-106, at a similar position in the C-terminal zinc finger, are defective for the ability to induce p53 degradation at any temperature (Fig. 3). Interestingly, recent analyses have shown that the temperature-sensitive mutants are unable to bind E6-AP at 37°C in vitro (12). It is likely that this phenotype is responsible for their inability to induce p53 degradation in vivo in MECs at early passage.

In conclusion, we show that HPV16 E6-induced p53 degradation is necessary for the immortalization of MECs. This observation is consistent with our previous finding that loss of p53 protein was an early event in radiation-induced transformation of MECs (35) and in a patient-derived breast tumor progression model (21). Importantly, p53 mutations are detected with high frequency in breast cancer patients (8, 27), and breast cancer-prone families with Li-Fraumeni syndrome have germ line p53 mutations (22, 34). Furthermore, recent studies by Shay et al. have shown spontaneous immortalization of MECs from patients with Li-Fraumeni syndrome (33). Taken together, these observations suggest that inactivation of p53 may represent an early and essential event during breast cell oncogenesis.

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