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hAda3 Degradation by Papillomavirus Type 16 E6 Correlates with Abrogation of the p14ARF-p53 Pathway and Efficient Immortalization of Human Mammary Epithelial Cells[▽]

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Two activities of human papillomavirus type 16 E6 (HPV16 E6) are proposed to contribute to the efficient immortalization of human epithelial cells: the degradation of p53 protein and the induction of telomerase. However, the requirement for p53 inactivation has been debated. Another E6 target is the hAda3 protein, a p53 coactivator and a component of histone acetyltransferase complexes. We have previously described the role of hAda3 and p53 acetylation in p14ARF-induced human mammary epithelial cell (MEC) senescence (P. Sekaric, V. A. Shamanin, J. Luo, and E. J. Androphy, Oncogene 26:6261–6268, 2007). In this study, we analyzed a set of HPV16 E6 mutants for the ability to induce hAda3 degradation. E6 mutants that degrade hAda3 but not p53 could abrogate p14ARF-induced growth arrest despite the presence of normal levels of p53 and efficiently immortalized MECs. However, two E6 mutants that previously were reported to immortalize MECs with low efficiency were found to be defective for both p53 and hAda3 degradation. We found that these immortal MECs select for reduced p53 protein levels through a proteasome-dependent mechanism. The findings strongly imply that the inactivation of the p14ARF-p53 pathway, either by the E6-mediated degradation of p53 or hAda3 or by cellular adaptation, is required for MEC immortalization.

Human papillomavirus type 16 (HPV16) causes ~50% of the cervical cancer burden. Its E6 and E7 proteins exhibit transforming properties through complex mechanisms. HPV16 E6 has been shown to induce p53 degradation and to stimulate the expression of human telomerase reverse transcriptase (hTERT). HPV16 E6 efficiently immortalizes primary mammary epithelial cells (MECs), but the contributions of p53 inactivation and hTERT activation remain controversial. In general, the immortalization of human epithelial cells has been associated with the activation of telomerase and the disruption of the p14ARF-p53 and p16-retinoblastoma pathways. Some primary human cells can be immortalized by the forced expression of hTERT alone, but these replicating cells select for reduced p14ARF and/or p16ink4a expression (8, 28, 32).

The activation of p53 can induce cell senescence, transient growth arrest, or apoptosis (reviewed in reference 13). p53 activation is manifested by the stabilization of the protein and complex posttranslational modifications, including acetylation and phosphorylation (reviewed in reference 3). Activated p53 regulates the transcription of several target genes, including p21cip1, and also has transcription-independent functions in apoptosis (reviewed in references 13 and 26). p53 acetylation is found during replicative and oncogene-induced senescence or stress-induced senescence. The major negative regulator of p53 is MDM2 (Hdm2 in human cells), which can ubiquitinate p53 and inhibit p53 acetylation (reviewed in reference 22). ARF (p14 in human cells, p19 in mouse cells) is a tumor suppressor that binds MDM2, inhibits MDM2 ubiquitin ligase function, stabilizes p53 (reviewed in reference 33), and induces p53 acetylation (20, 31).

Histone acetyltransferases (HATs) are essential components of eukaryotic transcription complexes. Apart from acetylating histones, several HATs (p300, CBP, PCAF, TIP60, and hMOF) acetylate p53 and function as p53 coactivators (reviewed in references 3 and 36). ADA3 (for alteration/deficiency in activation) is a component of yeast HAT complexes and is required for nucleosomal histone acetylation (1). Human Ada3 (hAda3) is a transcriptional coactivator of p53 as well as retinoic and estrogen receptors (15, 21, 37, 38). We recently reported that the RNA interference-mediated knockdown of hAda3 expression and truncated dominant-negative hAda3 abrogated the acetylation of lysine 382 in p53, inhibited p53 stabilization, and attenuated p14ARF-induced senescence (31).

We previously reported that E6 mutations at amino acids Phe 2 and Tyr 54 immortalized MECs but were incapable of inducing p53 degradation. Importantly, $E6^{Y54D}$-immortalized MECs are resistant to p14ARF-induced senescence despite normal levels of wild-type p53, which can be activated by DNA damage (32). $E6^{Y54D}$ induces the degradation of hAda3 (31), suggesting a mechanism for inhibiting p14ARF senescence signaling to p53 that is distinct from p53 degradation. These observations were consistent with the finding that the HPV16 E6 binding of hAda3 protein correlated with its ability to immortalize MECs (15).

Here, we sought to critically evaluate the correlation between E6-induced Ada3 degradation and the inhibition of p53

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TABLE 1. Phenotype of HPV16 E6 mutants

HPV16 E6 protein	p53 degradation in vivo	hADA3 degradation in vivo	p53 levels in MECs[e]		Induction of hTERT	Immortal MEC[g]
			Early passage	Late passage		
Wild type	+	+	Low[a]	Low[a]	+	6 (6)[a]
F2V	−	+	Normal[a]	Normal[a]	+	3 (4)[a]
Y54D	−	+	Normal[b]	Normal[b]	+	2 (2)
L37S	−	−	Normal[a]	Low[a]	+	1 (2)[a]
L110Q	−	−	Normal[a]	Low[a]	+	1 (2)[a]
G130V	−	−	Normal[c]	NA[f]	−[d]	0 (2)[c]
L50G	−	−	Normal[c]	NA	−[d]	0 (2)[c]

[a] Data are from reference 18.
[b] Data are from reference 32.
[c] Data are from Shamanin and Androphy, unpublished.
[d] Data are from reference 30.
[e] Normal indicates that the level was unchanged from that of the parental 76N primary MECs.
[f] NA, not applicable, since no viable late-passage cells were detected.
[g] Immortal MEC indicates the number of experiments in which the presence of immortal colonies was detected out of the total number of experiments performed (in parentheses).

activation by p14ARF. We tested a series of p53 degradation-defective HPV16 E6 mutants for hAda3 degradation and the inhibition of p14ARF-p53 signaling. We demonstrate that hAda3 degradation-competent E6 mutants block p14ARF-induced p53 acetylation and growth arrest and immortalize MECs. In contrast, E6 mutants defective for both p53 and hAda3 degradation are much less potent in inhibiting p14ARF-induced p53 activation. Notably, with this class of mutants, cells become immortal after a crisis period and display reduced levels of p53 protein. All MECs immortalized by E6 mutants express hTERT, implying that hAda3 degradation is not required for hTERT induction. In summary, we describe three subsets of p53 degradation-defective E6 mutants: those able to induce Ada3 degradation efficiently immortalized MECs; those unable to degrade Ada3 can lead to immortal MECs that avoid senescence by degrading p53; and those that do not degrade Ada3 and do not stimulate hTERT expression fail to immortalize. Thus, we have used HPV E6 to discern the requisite roles of hAda3 and cell adaptation in the inactivation of the p14ARF-p53 pathway during epithelial cell immortalization.

MATERIALS AND METHODS

Plasmids and cells. Plasmids, cells, and reagents and methods for cell culture, Western blotting, reverse transcription-PCR (RT-PCR), and flow cytometry were as described previously (31) unless otherwise noted. pcDNA3-p14ARF was provided by C. Sherr. pEGFPF, encoding farnesylated enhanced green fluorescent protein (EGFP), was from W. Jiang. HPV16 E6 mutants in pLXSN were described previously (18). The phenotypes of HPV16 E6 mutants are shown in Table 1. pBabe-puro-11E6 was from L. Gustavo. pLXSN-6bE6 was provided by M. Tommasino. The E6 sequence from the HPV6vc plasmid was amplified by PCR and cloned into pLXSN, and the sequence was confirmed. HPV6vc E6 differs from HPV6b E6 by having a glutamine at position 50 instead of a histidine.

Antibodies. The following antibodies were used: p14ARF (14P03; 1:100; Neomarkers), p53 DO-1 (1:500; Santa Cruz), FL393 (1:500; Santa Cruz), p21cip1 (SX118; 1:200; Pharmingen), Flag (M2; 1:3,000; Sigma), HDM2 (clone IF2; Ab-1; 1:400; Oncogene Research), actin (A2066; 1:4,000; Sigma), goat anti-mouse horseradish peroxidase (1:4,000; Jackson), goat anti-rabbit horseradish peroxidase (1:4,000; Jackson), and K382-acetylated p53 (2525; 1:1,000; Cell Signaling).

Treatment with MG132. For studies with the proteasome inhibitor MG132, 10^6 cells were seeded in 100-mm dishes and grown for 24 h. MG132 in dimethylsulfoxide (DMSO) was added to a 10 μM concentration, and the cells were incubated for 4 h.

RT-PCR. HPV16 E6 RNA was detected by RT-PCR using primers 16E6S (dAAGCAACAGTTACTGCGACGTGAG) and 16E6A (dCGGTCCACCGA CCCCTTATATT). E6 mRNA of different HPV types was detected by RT-PCR using the following primers to LXSN sequences flanking the E6 cloning site: pLXSN1S (dTTTAACCGAGACCTCATCACC) and pLXSN1A (dCCACACC CTAACTGACACACA).

RESULTS

HPV16 E6^{Y54D} inhibits p14ARF-induced p53 acetylation. MECs transduced by exogenous hTERT select for the down-regulation of p14ARF expression during serial passage (32). These cells are susceptible to p53-dependent senescence by ectopic p14ARF. In contrast, E6^{Y54D}-immortalized MECs constitutively express p14ARF and undergo transient growth arrest but not senescence in response to p14ARF (32). Since E6^{Y54D} does not degrade the p53 protein, the E6 inhibition of p14ARF senescence signals and E6-mediated p53 degradation are distinguishable.

Since E6^{Y54D} degrades Ada3, we predicted it would interfere with the p14ARF-induced stabilization and acetylation of p53 in MECs. To test this, late-passage hTERT or E6^{Y54D}-immortalized MECs were infected with p14ARF-expressing retroviruses. At day 7 postinfection, when hTERT MECs displayed the senescent phenotype (32), the levels of total p53 and lysine 382-acetylated p53 were analyzed. Ectopic p14ARF induced the accumulation of total and K382-acetylated p53 in hTERT MECs (Fig. 1A, lanes 1 and 2), but this response was effectively blocked in E6^{Y54D} MECs (lanes 5 and 6). To prove that this was not a clonal effect specific for E6^{Y54D} MECs, late-passage hTERT MECs were infected with recombinant E6^{Y54D}-expressing retroviruses, and the selected population was challenged with p14ARF. Consistently with the prior experiments, E6^{Y54D} inhibited p53 stabilization (Fig. 1, lanes 3 and 4). These data demonstrate that in MECs, HPV16 E6 can inhibit p53 activation induced by p14ARF independently of p53 degradation.

We sought to confirm the effects of E6 in another cell model. U2OS osteosarcoma cells are p14ARF deficient and express wild-type p53, so they are commonly used to study p53 and p14ARF functions (34). In contrast to primary MECs or hTERT MECs, U2OS cells are readily transfectable, and the

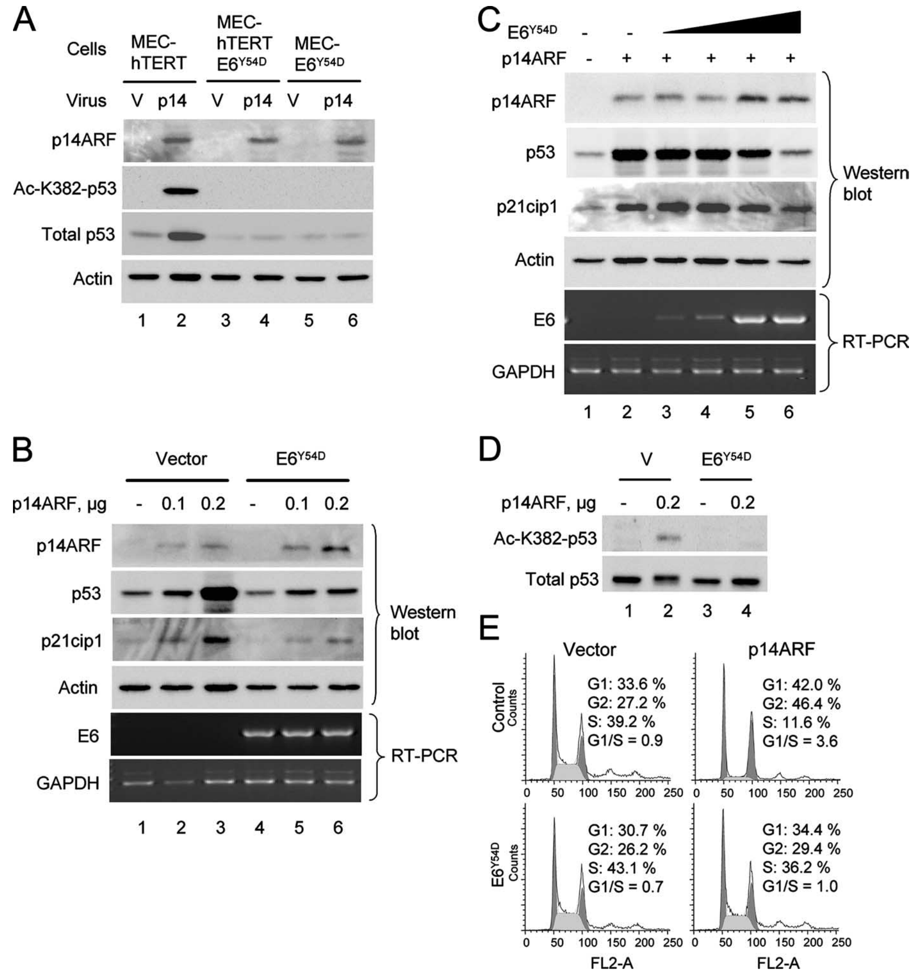

FIG. 1. E6^{Y54D} is defective in p53 degradation and inhibits p53 acetylation, stabilization, and growth arrest induced by p14ARF. (A) hTERT MECs, E6^{Y54D} MECs, and hTERT MECs expressing E6^{Y54D} were infected with pWZL-hygro-p14ARF or control pWZL-hygro retrovirus, selected with hygromycin, and harvested at day 7 postinfection. Cell lysates corresponding to 30 μg protein were analyzed by Western blotting. Actin was used as a loading control. Note that E6^{Y54D} inhibits p53 stabilization induced by p14ARF. (B) U2OS cells were transfected with increasing amounts of pcDNA3-p14ARF and 1 μg pLXSN-16E6^{Y54D} or vector control. Cells were harvested 48 h posttransfection. p14ARF, p53, and p21cip1 proteins were detected by Western blotting, and E6 mRNA was detected by RT-PCR. Actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as loading controls. In U2OS cells, p14ARF induced the dose-dependent stabilization of p53 that was inhibited by E6^{Y54D}. (C) U2OS cells were transfected with 0.2 μg pcDNA3-p14ARF and 0.01, 0.05, 0.2, or 1.0 μg pLXSN-16E6^{Y54D}. Cells were harvested 48 h posttransfection. The expression of p14ARF, total p53, p21cip1, and E6^{Y54D} was detected as described for panel B. E6^{Y54D} inhibited p14ARF-induced p53 stabilization and the induction of p21cip1. (D) Cell lysates from panel B were normalized for total p53, and the acetylation of lysine 382 in p53 was detected by Western blotting (lanes 1 and 3, 28 μg protein; lane 2, 1.8 μg protein; lane 4, 20 μg protein). The amount of HPV16 E6^{Y54D} or empty vector (V) in the transfection was 1 μg. (E) U2OS cells were transfected with 0.5 μg pEGFPF+, 0.2 μg pcDNA3-p14ARF, and 1 μg pLXSN-E6^{Y54D} or empty vectors. Monolayer cells were harvested 48 h posttransfection and stained with propidium iodide, and the DNA content of EGFP-positive cells was analyzed by FACS.

forced expression of p14ARF in U2OS cells induces growth arrest but not senescence (19, 34). As predicted, the transfection of p14ARF into U2OS cells stabilized p53 and induced p21cip1 in a dose-dependent manner (Fig. 1B, lanes 1 to 3). Importantly, cotransfection with E6^{Y54D} abrogated this response (lanes 4 to 6). The E6-induced inhibition of p53 stabilization was dose dependent (Fig. 1C, lanes 3 to 6), and p14ARF-induced p53 stabilization (lanes 1 to 2) was virtually abolished at the highest input level of E6^{Y54D} (lane 6). We noticed that, in these experiments, E6^{Y54D} reproducibly increased the levels of the exogenous p14ARF protein (Fig. 1B, lanes 3 and 6, and C, lanes 2, 5, and 6). p53 was proposed to inhibit p14ARF expression by an unknown posttranscriptional mechanism (34), and E6^{Y54D} may release p14ARF from p53 repression.

Like wild-type E6, E6^{Y54D} induces the degradation of hAda3, which we have implicated in the acetylation and activation of p53 by p14ARF (31). We therefore reasoned that E6^{Y54D} would inhibit p53 activation by blocking p53 acetylation. Since differences in the total p53 levels also could contribute to apparent changes in p53 acetylation, we first normalized U2OS lysates for an input of total p53 and then detected K382-acetylated p53 in repeated Western blottings (Fig. 1D). As expected, p14ARF induced the acetylation of p53. Remarkably, this was completely blocked by E6^{Y54D}, in agreement with observations for MECs.

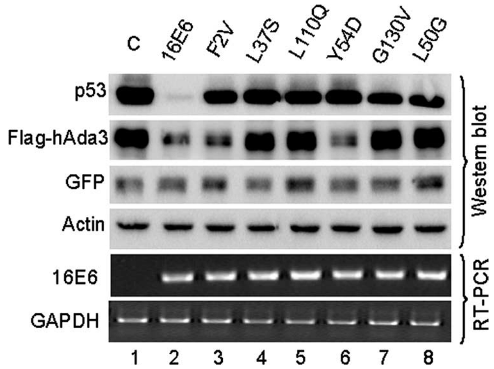

FIG. 2. hAda3 and p53 degradation by HPV16 E6 (16E6) mutants in vivo. H1299 cells were transfected with pcDNA3-Flag-hAda3 (1 μg), pCMV-p53 (1 μg), and pLXSN-HPV16 E6 DNA (3 μg). Cells were harvested 48 h posttransfection. The levels of p53 and Flag-hAda3 proteins were analyzed by Western blotting, and E6 mRNA was analyzed by RT-PCR. Actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as loading controls. C, pLXSN vector control.

Since p14ARF induces p53-dependent growth arrest in U2OS cells (34), we tested the impact of $E6^{Y54D}$ on this response. In agreement with the Western blotting data, cell cycle analysis by a fluorescence-activated cell sorter (FACS) showed that $E6^{Y54D}$ inhibited p14ARF-induced G_1-phase growth arrest (Fig. 1E). Taken together, the results of these experiments document that in mammary epithelial and U2OS cells, the p53 degradation-defective mutant $E6^{Y54D}$ inhibits p14ARF-induced p53 acetylation, p53 stabilization, p21cip1 induction, and growth arrest.

E6-induced degradation of Ada3 correlates with efficient MEC immortalization but is not absolutely required. Based on the results described above, we considered that HPV16 E6-mediated hAda3 degradation represents the immortalization activity of p53 degradation-defective HPV16 E6 mutants. We attempted to detect levels of endogenous hAda3 protein in the E6-immortalized MECs by using hAda3 antisera (24, 31); however, this was unsuccessful, probably due to the very low levels of endogenous hAda3 protein (data not shown). Therefore, we used the established cotransfection assay to evaluate the ability of these HPV E6 mutants to induce p53 and hAda3 degradation in vivo (15, 18). HPV16 E6 was cotransfected with Flag-hAda3 or p53 expression plasmids into the p53 null cell line H1299. $E6^{F2V}$ and $E6^{Y54D}$ reduced Flag-hAda3 protein levels to extents similar to those of wild-type HPV16 E6 but did not reduce p53 levels (Fig. 2). These results are in agreement with those of an earlier study that also used $E6^{F2V}$ and $E6^{Y54H}$ (15). Therefore, these E6 mutants reduce hAda3 levels, efficiently immortalize MECs, and maintain normal p53 levels. For comparison, we selected mutants $E6^{L37S}$ and $E6^{L110Q}$, which have low levels of immortalizing activity (18), and $E6^{L50G}$ and $E6^{G130V}$, which did not immortalize MECs (Table 1). These E6 mutants did not induce p53 or hAda3 degradation in H1299 cells (Fig. 2). Since the expression levels of HPV16 E6 mutations might influence hAda3 degradation, we used RT-PCR to detect HPV16 E6 mRNA and observed no differences in their expression levels (lanes 2 to 8). In summary, the E6 mutants that induce p53 or hAda3 degradation efficiently immortalize MECs (Table 1). In contrast, $E6^{L37S}$ and $E6^{L110Q}$, which were unable to degrade these E6 target proteins, rarely produced immortal cells.

E6-induced degradation of hAda3 correlates with inhibition of p14ARF-induced p53 activation and growth arrest. We further tested the effects of these E6 mutants on p14ARF-induced p53 activation and growth arrest in U2OS cells. The levels of p53 and p21cip1 proteins increased in U2OS cells following p14ARF transfection (Fig. 3A, lanes 1 and 2). Wild-type HPV16 decreased the steady-state levels of p53 and abolished the p14ARF induction of p53 and p21cip1 (lanes 3 and 4). $E6^{F2V}$, which is phenotypically similar to $E6^{Y54D}$ in inducing hAda3 degradation, abrogated p53 stabilization and the induction of p21cip1 (lanes 5 and 6). In contrast, cells expressing $E6^{L110Q}$, $E6^{G130V}$, and $E6^{L50G}$, which did not degrade hAda3, increased p53 levels (lanes 8, 10, and 12). Compared to that of the control (lane 1), $E6^{G130V}$ and $E6^{L110Q}$ exhibited decreased basal levels of p21cip, while $E6^{L50G}$ showed no reduction (lanes 7, 9, and 11). In response to p14ARF, these three mutations showed inductions of p21 levels, with $E6^{L50G}$-transfected U20S cells exhibiting the greatest increase in p21 protein levels (Fig. 3A, lanes 11 and 12). Taken together, these data reveal that the abrogation of p14ARF-induced p53 activation correlates with the ability of E6 to induce hAda3 degradation.

We then investigated the impact of these E6 mutants on p53 acetylation. Since it was possible that acetylation was not detectable in the cells with low levels of p53, the input protein was adjusted to normalize total p53 protein levels, and p53 acetylation at lysine 382 was retested (Fig. 3B). The hAda3 degradation-competent mutants $E6^{F2V}$ (Fig. 3B) and $E6^{Y54D}$ (Fig. 1A) completely blocked p53 acetylation by K382. In contrast, $E6^{L110Q}$, $E6^{G130V}$, and $E6^{L50G}$, which do not degrade hAda3, inhibited p53 acetylation by K382 by about 60%. Interestingly, while the percentages of p53 that are acetylated on K382 in $E6^{L110Q}$ and $E6^{L50G}$ cells were similar, the L110Q mutant had lower levels of total p53 and, hence, fewer acetylated p53 molecules, corresponding to the reduced expression of p21cip1 (Fig. 3A, compare lanes 7 and 8 to 11 and 12). Moreover, compared to that of the control, $E6^{L50G}$ did not inhibit the accumulation of p53 or p21cip1 in response to p14ARF (Fig. 3A, compare lane 2 to 12).

Using flow cytometry to analyze the cell cycle distribution of transfected U20S cells, we found that wild-type HPV16 as well as $E6^{F2V}$ blocked p14ARF-induced growth arrest (G_1/S ratios of 1.1 to 1.5) (Fig. 3C). $E6^{L110Q}$ was less efficient (G_1/S ratio of 3.4), while $E6^{G130V}$- and $E6^{L50G}$-expressing cells demonstrated profound G_1 arrest, with G_1/S ratios of 8.8 and 10.9, respectively. In summary, these experiments demonstrate that hAda3 degradation by HPV16 E6 correlates with the abrogation of p14ARF-induced p53 acetylation and the inhibition of p53 stabilization and growth arrest.

Low-risk genital HPV6 and HPV11 E6 do not induce hAda3 degradation and do not inhibit p14ARF-p53 signaling. Low-risk genital HPV6 and HPV11 are associated with benign hyperproliferative anogenital and cervical lesions (reviewed in reference 40). However, in rare cases these types are detected in malignant lesions, and HPV6vs was isolated from invasive vulvar carcinoma (27). There are divergent interpretations regarding whether low-risk E6 proteins alter p53-dependent re-

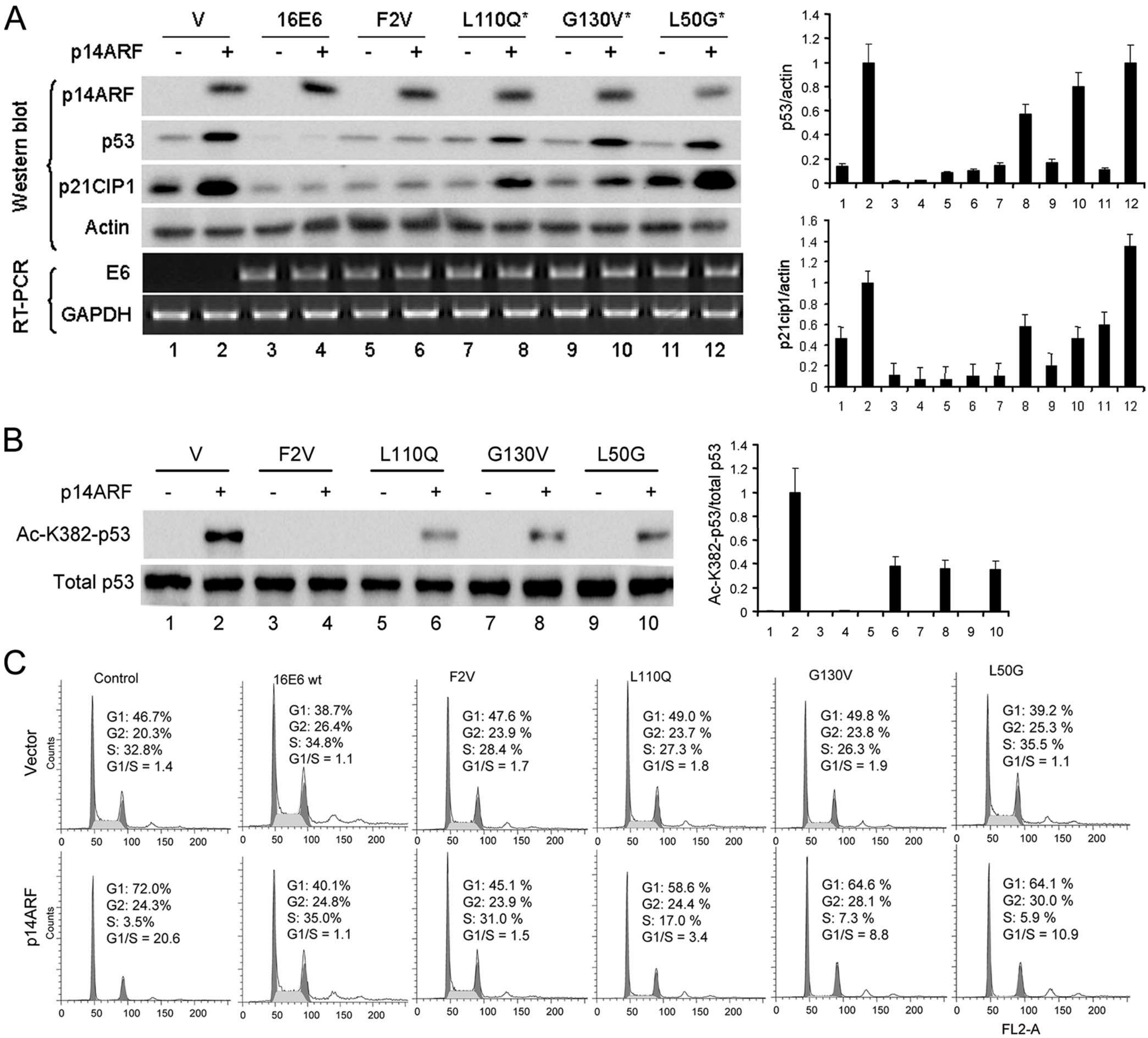

FIG. 3. Inhibition of p14ARF-induced p53 activation and growth arrest by HPV16 E6 (16E6) mutants that are defective in p53 degradation. (A) U2OS cells were transfected with 0.2 μg p14ARF and 1 μg wild-type or mutant HPV16 E6 or empty vector DNA (V). The expression of p14ARF, p53, and p21cip1 proteins and HPV16 E6 mRNA was detected as described in the legend to Fig. 1B. Asterisks indicate HPV16 E6 mutants defective in both p53 and hAda3 degradation. The graph shows the levels of p53 and p21cip1 quantified using an LAS1000+ luminescent image analyzer (Fuji) and normalized to actin. Levels in control cells transfected with p14ARF (lane 2) were set to 1. Note that E6 and $E6^{F2V}$ completely blocked the p14ARF-induced accumulation of p53 and p21cip1. (B) Cell lysates from the experiments shown in panel A were normalized for total p53, and the acetylation of lysine 382 in p53 was detected as described in the legend to Fig. 1D. The graph shows the levels of acetylated p53 that were quantified and normalized to total p53 as described for panel A. (C) U2OS cells were transfected with GFP-, p14ARF-, and HPV16 E6-expressing plasmids or control vectors (V). The DNA content of propidium iodide-stained cells was determined by FACS as described in the legend to Fig. 1E. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ac-K382-p53, K382-acetylated p53.

sponses. Low-risk HPV E6 proteins do not induce p53 degradation (6, 12, 29), and HPV6b E6 did not bind hAda3 (15). However, HPV11 E6 blocked the UV induction of p21cip1 while $16E6^{L37S}$ did not (35), suggesting that HPV11 E6 inhibits p53 activation. We tested low-risk E6 for hAda3 degradation and the inhibition of p14ARF-induced p53 activation. The expression of HPV6b, HPV6vc, or HPV11 E6 in H1299 cells did not induce the degradation of Flag-hAda3 and p53 (Fig. 4A). Furthermore, the expression of these E6 types did not affect p53 stabilization and p21cip1 induction by p14ARF in U2OS cells (Fig. 4B). In the same experiment, HPV16 $E6^{F2V}$ blocked the accumulation of p53 and p21cip1. Since the inhibition of p53 stabilization by E6 is dose dependent (Fig. 1C), we verified the expression of E6 mRNA by RT-PCR. These data suggest that low-risk HPV E6 proteins do not target hAda3 and do not inhibit p14ARF signaling to p53.

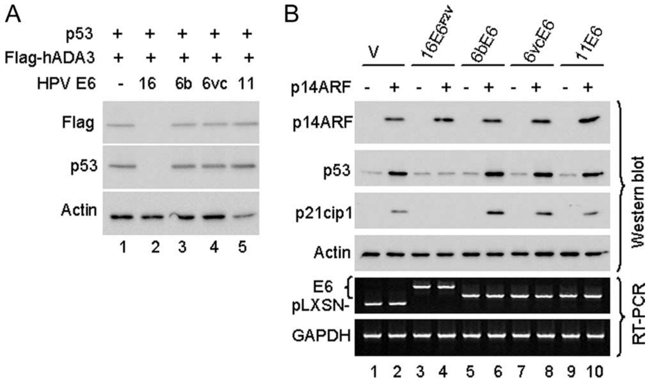

FIG. 4. Low-risk genital HPV E6s do not induce the degradation of hAda3 and do not inhibit the p14ARF-induced accumulation of p53 and p21cip1. (A) H1299 cells were transfected with Flag-tagged hAda3, p53, and HPV6b, HPV6vc, HPV11, or HPV16, as described in the legend to Fig. 2. The expression of Flag-hADA3 and p53 was tested by Western blotting. Note that, in contrast to HPV16 E6, the HPV6 and HPV11 E6 do not induce hAda3 degradation. (B) U2OS cells were transfected with p14ARF, HPV6b E6, HPV6vc E6, HPV16 $E6^{F2V}$, or empty vector (V), as described in the legend to Fig. 3A. The expression of p14ARF, p53, and p21cip1 proteins was detected by Western blotting, and E6 mRNA was detected by RT-PCR. Note that HPV6 E6 and HPV11 E6 do not inhibit the accumulation of p53 and p21cip1 proteins induced by p14ARF. HPV16 $E6^{F2V}$ was used for comparison. Actin was used as a loading control.

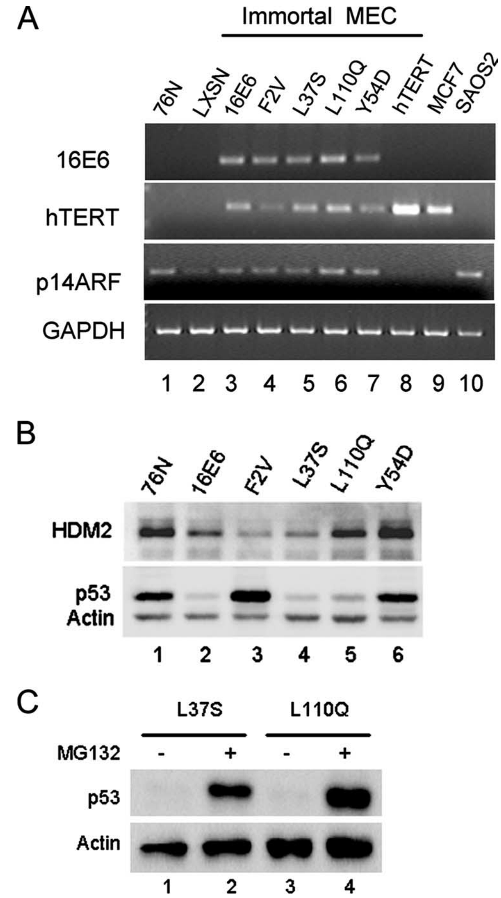

FIG. 5. MECs immortalized by hAda3 and p53 degradation-defective E6 mutants maintain the expression of p14ARF but select for the proteosomal degradation of the p53 protein. (A) The expression of p14ARF, hTERT, and HPV16 E6 mRNA was tested by RT-PCR in parental 76N primary MECs, cells infected with pLXSN vector, or late-passage HPV16 E6-immortalized MECs (lanes 3 to 7). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control. hTERT-immortalized MECs (lane 8) were used for comparison. MCF7 and SAOS2 cell lines were used as PCR standards for hTERT and p14ARF, respectively. MCF7 is a p14ARF-negative, telomerase-positive breast cancer cell line; SAOS2 is a p14ARF-positive, telomerase-negative osteosarcoma cell line. Note that the hTERT-immortalized MECs down-regulate p14ARF compared to the regulation of p14ARF by parental primary 76N MECs. In contrast, 76N MECs immortalized by wild-type and mutant HPV16 E6 activate hTERT expression and maintain p14ARF. (B) Cell lysates corresponding to 30 μg protein were tested by Western blotting for p53 and HDM2 proteins. Actin was used as a loading control. In comparison to $E6^{F2V}$- or $E6^{Y54D}$-immortalized MECs, the $E6^{L37S}$ and $E6^{L110Q}$-immortalized cells exhibit reduced p53 protein levels. Parental primary 76N MECs and wild-type HPV16 E6-immortalized MECs were included for comparison. Note that $E6^{L37S}$- and $E6^{L110Q}$-immortalized MECs did not overexpress HDM2. (C) Western blotting of $E6^{L37S}$- and $E6^{L110Q}$-immortalized MECs treated for 4 h with DMSO (lanes 1 and 3) or 10 μM proteasome inhibitor MG132 (lanes 2 and 4). Actin was used as a loading control. 16E6, HPV16 E6.

E6-immortalized MECs maintain p14ARF expression. We reasoned that since $E6^{F2V}$ and $E6^{Y54D}$ induce hAda3 degradation and interfere with p14ARF senescence signals, the respective immortal cells maintain p14ARF expression. For comparison purposes, we included MECs immortalized by $E6^{L37S}$ and $E6^{L110Q}$, which did not induce hAda3 and p53 degradation, as well as MECs immortalized by the forced expression of hTERT. Interestingly, RT-PCR revealed that all MEC lines immortalized by these E6 mutants maintained the expression of p14ARF mRNA similarly to the parental 76N cells (Fig. 5A) irrespective of the ability to degrade hADA3. In contrast, hTERT MECs dramatically down-regulated p14ARF, in accordance with our previous report (32). Of note, hTERT mRNA expression was elevated in all HPV16 E6-immortalized MECs.

MECs immortalized by hAda3 and p53 degradation-defective E6 mutants select for proteosomal degradation of the p53 protein. One critical difference between $E6^{L50G}$ and $E6^{G130V}$, which are immortalization defective, and $E6^{L37S}$ and $E6^{L110Q}$, which are immortalization competent, is that the latter pair maintains the ability to stimulate the expression of hTERT (30), although all four are unable to degrade hAda3. The question remained how these hAda3 and p53 degradation-defective mutants evade replicative senescence, as they were unable to abrogate p14ARF signaling to p53. The clue was that while $E6^{L37S}$ and $E6^{L110Q}$ were unable to acutely induce p53 degradation in MECs, after a latency period in culture, a MEC population spontaneously arose and proliferated in growth factor-deficient media for multiple population doublings (18). As we reported then and confirmed here, compared to those of parental primary 76N cells, $E6^{L37S}$- and $E6^{L110Q}$-immortalized MECs expressed very low levels of p53 protein, levels that were comparable to those of wild-type HPV16 E6-immortalized MECs (Fig. 5B). We also confirmed that the immortal cells maintain the $E6^{L37S}$ and $E6^{L110Q}$ mutations and express normal levels of p53 mRNA (data not shown). This fact implies that cells immortalized by $E6^{L37S}$ and $E6^{L110Q}$ bypass the senescence pathway by secondary events selecting for accelerated p53 protein degradation. Multiple factors are known to regulate p53 turnover, one of which is the ubiquitin ligase Hdm2, which is overexpressed in a proportion of human tumors, including breast cancers (23). However, Hdm2 protein levels were not increased in the $E6^{L37S}$- and $E6^{L110Q}$-immortalized cells (Fig. 5B). Notably, the exposure of these cells to the proteasome inhibitor MG132 resulted in a robust increase in p53 protein (Fig. 5C), implying its enhanced turnover in the immortal cells by the ubiquitin/proteasome pathway.

DISCUSSION

We previously reported that MECs were efficiently immortalized by HPV16 $E6^{Y54H}$ and $E6^{F2V}$, although p53 protein levels were unchanged compared to those of the parental primary cells (18). Based on these results, we concluded that E6-mediated p53 degradation was not strictly necessary for immortalization. In the same study, $E6^{L37S}$ and $E6^{L110Q}$ were similarly acutely defective for p53 degradation following the retroviral infection of MECs and in reticulocyte lysate assays. However, the few MEC colonies that expressed $E6^{L37S}$ and $E6^{L110Q}$ that became immortal expressed levels of p53 protein equal to that of MEC colonies that expressed wild-type E6, implying selective pressure to abrogate senescence signaling by p53. Here, we reconcile these seemingly discordant observations and further elucidate the underlying mechanisms that allow continuous cell proliferation in culture.

The signaling of p14ARF to p53 is activated in response to oncogenic stress and results in cell senescence (33). p14ARF-induced senescence is accompanied by p53 acetylation and requires the hAda3 protein (31), which complexes with p53 and p300 (15, 37). Previously, we reported that the p53 degradation-defective mutant HPV16 $E6^{Y54D}$ inhibited p14ARF-induced senescence (32). We now demonstrate that E6 mutants capable of hAda3 degradation but that do not induce p53 degradation also block p14ARF-induced p53 acetylation and activation in MECs and U20S cells. Taken together with the fact that dominant-negative hAda3 and hAda3 RNA interference inhibit p53 acetylation and p14ARF-p53 signaling (31), our data strongly imply that the E6 degradation of hAda3 is sufficient to block p14ARF-induced senescence. The inactivation of the p14ARF and p53 tumor suppressors is a frequent event in cancer. The ability of high-risk HPV E6 but not low-risk HPV E6 to induce hAda3 degradation therefore is likely to contribute to the oncogenic properties of these viruses.

E6-induced degradation of hAda3 represents one mechanism to inhibit p53 acetylation at lysine 382 and the subsequent attenuation of p53 function. However, since hAda3 degradation-defective mutants such as $E6^{L50G}$ also reduced p53 acetylation, albeit less efficiently, other mechanisms may exist. This notion is supported by observations that HPV16 E6, including mutants such as $E6^{L50G}$, bind the HATs CBP/p300 and inhibit their transcriptional activation activity (25, 39). On this basis, E6 mutants unable to target Ada3 for degradation also may interfere with p53 acetylation by HATs. While hAda3 is a component of multiple HAT complexes, it remains to be determined which HAT is specifically involved in p53 acetylation in response to p14ARF.

In this study, we found that E6-mediated hAda3 degradation blocked p14ARF-induced p53 stabilization, which correlated in part with the inhibition of p53 acetylation by K382. However, the interplay between p53 acetylation and p53 stabilization is complex. $E6^{L50G}$ inhibited p53 acetylation by about 60%, but p53 stabilization induced by p14ARF was not affected. In accord with this observation, the replacement of several C-terminal lysine residues by arginine, which cannot be acetylated, had only mild effects on p53 stabilization and function (9, 14). Interestingly, both $E6^{L110Q}$ and $E6^{L50G}$ reduced p53 acetylation; however, the former more efficiently inhibited p53 stabilization and growth arrest following p14ARF expression than the latter. It appears that p53 acetylation is associated with but is not equal to p53 activity, since the latter depends not only on p53 acetylation but also on other p53 modifications (e.g., phosphorylation) and the status of the other p53-interacting proteins. It is quite likely that, apart from regulating the activity of HAT(s) toward p53, hAda3 and E6 regulate acetylation and functions of other proteins, such as protein kinases, that are involved in p53 phosphorylation. Interestingly, p14ARF-induced growth arrest is dependent on the ATM kinase (17) that phosphorylates p53 at serine 15, and hAda3 is required for serine 15 phosphorylation (24 and V. Shamanin and E. J. Androphy, unpublished data). Further mechanistic studies of hAda3 and the role of E6 in p14ARF-p53 signaling are warranted to explore these intriguing possibilities.

While the role of high-risk E6 in cell transformation is established, the functions of E6 proteins from low-risk genital and cutaneous HPVs remain unknown. Low-risk E6 proteins do not degrade p53 (6, 29) and have weak immortalizing activity in human epithelial cells (2, 11). HPV6 E6 did not bind hAda3 (15) and did not inhibit DNA damage-induced p53 stabilization and growth arrest (10). In agreement with these data, we found that HPV6 and HPV11 E6 did not induce hAda3 degradation and did not alter the p14ARF-induced accumulation of p53 and p21cip1, implying that low-risk E6 does not affect p53 activation. In contrast, recently it was reported that HPV11 E6 inhibited the p53 activation of the p21cip1 promoter in vitro and in vivo (35). This disagreement could be explained by their use of UV light and the stimulation of the DNA damage response, while our studies used p14ARF. Interestingly, cutaneous HPV38 E6 does not degrade p53 but cooperates with E7 in the immortalization of human keratinocytes (4, 5). We presently are testing cutaneous E6 proteins for the ability to inhibit the p14ARF-induced senescence and a potential role in hAda3 inactivation.

Two mutants previously reported to immortalize MECs with low efficiency, $E6^{L37S}$ and $E6^{L110Q}$, were defective for both p53 and hADA3 degradation. Importantly, $E6^{L37S}$ and $E6^{L110Q}$ retain the full capability to induce hTERT early after expression in human foreskin keratinocytes (30). Therefore, p53 and hAda3 degradation is not required for hTERT induction by E6. Furthermore, inefficient immortalization by hAda3 and p53 degradation-defective E6 mutants is not due to a defect in hTERT activation but rather to their inability to block the p14ARF-p53 pathway. This is supported by the observation that rare clones of $E6^{L37S}$ and $E6^{L110Q}$ MECs that escaped senescence have selected for low p53 levels. Interestingly, p53 levels in $E6^{L37S}$- and $E6^{L110Q}$-immortalized MECs were restored by MG132, implying the enhanced proteosomal degradation of p53. Our data illustrate three mechanisms of the abrogation of the p14ARF-p53 pathway associated with the immortalization of MECs. First, the serial passage of hTERT-transduced MECs selects for cells with a reduced expression of p14ARF mRNA in the presence of wild-type levels of p53 (32). These cells remain susceptible to p14ARF-induced senescence. Second, cells expressing E6 mutants such as F2V or Y54D that are capable of degrading hADA3 maintain normal levels of p53 protein, but p53 activation and acetylation by p14ARF are blocked. Third, cells with E6 mutant L37S or L110Q, defective for p53 and hADA3 degradation, avoid replicative senescence by cellular adaptation through the increased turnover of p53 protein. In the latter two conditions, the immortal cells maintain endogenous p14ARF expression, because levels of the downstream effector hAda3 or p53 are reduced (32). Several cellular E3 ligases regulate the proteosomal degradation of the p53 protein (13). Hdm2 is the major E3 ligase involved in p53 regulation, and the overexpression of Hdm2 was implicated in p53 inactivation in some human cancers (23). However, $E6^{L37S}$- and $E6^{L110Q}$-immortalized MECs expressed reduced levels of Hdm2 compared to those of parental MECs, implying that these cells degrade p53 by activating another E3 ligase. COP1 and Pirh2 are reasonable candidates, as these are overexpressed in breast and lung cancers (7, 16). Further studies are necessary to identify the p53 ubiquitin ligase upregulated in $E6^{L37S}$- and $E6^{L110Q}$-immortalized MECs, since this may reveal a molecular marker of the predisposition to and the mechanism of malignant transformation in breast cancer.

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