Type-specific interaction between human papillomavirus type 58 E2 protein and E7 protein inhibits E7-mediated oncogenicity

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Human papillomavirus type 58 (HPV-58) is a very common HPV type in eastern Asia. Little is known about its biology and tumorigenesis. In this study, HPV-58 E2 protein (58E2) was found to interact with E7 protein (58E7), and the hinge domain of 58E2 was shown to be responsible for binding to the 58E7 protein. Interestingly, the E2–E7 interaction appears to be HPV type-specific, as we found that the HPV-16 E2 could not bind to the 58E7 protein, and neither did 58E2 interact with HPV-16 E7. The biological consequence(s) of the E2–E7 interaction in HPV-58, especially in viral tumorigenesis, was investigated. Results showed that, through interacting with 58E7, 58E2 prevented E7-induced retinoblastoma protein (pRb) degradation and prolonged the half-life of pRb in cells. Additionally, 58E2 abrogated 58E7-induced cell proliferation. These observations collectively suggest that direct interaction with 58E7 is another mechanism for 58E2 to inhibit 58E7-associated carcinogenesis in addition to regulating expression of the 58E7 gene.

INTRODUCTION

Human papillomavirus (HPV) infects mucosal epithelial tissue at different anatomical locations, resulting in a variety of clinical manifestations ranging from benign warts to invasive genital cancers (Doorbar, 2005; Howley & Lowy, 2001). The high-risk HPV types are commonly associated with lesions that can progress into cervical cancer, which is one of the leading causes of cancer death in women (zur Hausen, 2002). The carcinogenicity of high-risk HPVs mainly depends on the activities of two virally encoded oncoproteins, namely E6 and E7. They bind at a high affinity and degrade tumour suppressors p53 and the retinoblastoma protein (pRb), respectively (Boyer et al., 1996; Dyson et al., 1989; Scheffner et al., 1990).

In low-grade squamous intraepithelial lesions (LSILs), the HPV genomes replicate episomally and the levels of E6 and E7 expression are low. In contrast, high-grade squamous intraepithelial lesions (HSILs) and squamous cell carcinoma of the cervix (SCC) often show high-level expression of E6 and E7. In most cases of HSIL and SCC, the HPV genomic DNA is integrated into the host-cell genome (von Knebel Doeberitz, 2002), which is always associated with the interruption of E2 ORF and loss of the E2 protein (Corden et al., 1999). The correlation of elevated levels of E6 and E7 expression and the frequent integration of the viral genome in a manner that disrupts E2 gene expression integration suggests a role of the E2 protein in negative regulation of viral oncogenic proteins. It is known that one of the negative-regulation mechanisms is that the C-terminal domain of the E2 protein (E2CT) binds to the E6/E7 promoter at the E2-binding sites (E2BS) and regulates E6/E7 expression (Androphy et al., 1987). Recently, it was reported that the HPV type 16 (HPV-16) E2 protein interacts directly with the E7 protein (Gammoh et al., 2006) and inhibits E7-induced pRb degradation and cell transformation (Gammoh et al., 2009). Thus, in addition to suppressing E7 expression, the HPV-16 E2 protein can also inhibit the oncogenic activity of E7 by direct protein–protein interaction.

HPV type 58 (HPV-58) is the third most common HPV type in Eastern Asia (Parkin et al., 2008). Recently, the largest assessment to date showed that HPV-58 causes around 2% of cases of invasive cervical cancer worldwide, while in Asia it causes 4% of cases, following HPV-16 (60%), HPV-18 (11%) and HPV-52 (6%) (de Sanjose et al., 2010). However, it is found that the prevalence of HPV-58 infection is much higher in Eastern Asia. A recent report showed that HPV-16 (26.5%), HPV-58 (8.8%) and HPV-33 (7.8%) were the most prevalent types in cervical lesions including LSILs, HSILs and SCC in Beijing, China (Li et al., 2010). As the HPV-16/18 L1 vaccine cannot protect against HPV-33, HPV-52 and HPV-58 (Harper et al., 2006), it is foreseen that, following vaccination with the HPV-16/18 L1 vaccine, the incidence of cervical lesions
caused by other high-risk HPVs, including HPV-33, HPV-52 and HPV-58, might increase in the future. However, little effort has been spent on exploring the biology and tumorigenesis of HPV-58, especially with regards to the function of the HPV-58 E2 protein (58E2). In the current study, we investigated the interaction between the 58E2 and HPV-58 E7 (58E7) proteins, and the functional consequence of the interaction. We found that 58E2 binds to 58E7, while there is no cross interaction between HPV-16 E7 protein (16E7) and 58E2, or between HPV-16 E2 protein (16E2) and 58E7. Furthermore, 58E2 is found to block the transformation of 58E7 by preventing 58E7-mediated degradation of pRb.

RESULTS

58E2 protein binds 58E7 protein in vitro

It has been shown that E2 suppresses cellular growth through repressing viral E6 and E7 expression at the transcription level (Goodwin & DiMaio, 2000; Dowhanick et al., 1995). Recent studies suggest that E2 may regulate E6 and E7 functions at an additional level. The E2 protein of HPV-16 (16E2) was found to directly interact with the E7 protein (16E7) (Gammoh et al., 2006). We were wondering whether the interaction of the E2 and E7 proteins is a unique feature to HPV-16 or a general phenomenon in all types of HPV. To this end, we examined the interaction of E2 and E7 proteins in HPV-58 (58E2 and 58E7). A glutathione S-transferase (GST) pull-down assay was first employed. Because of the difficulty in expressing full-length 58E2 in Escherichia coli, the N-terminal domain (NT, 1–201 aa) and the C-terminal domain (HDCT2, 225–358 aa) of 58E2 were cloned separately in a GST vector and the corresponding GST-tagged fragments were expressed respectively in E. coli. The 58E7 protein was also cloned and expressed as a fusion protein with a 6 x His tag. The purified GST–58E2 truncations and His–58E7 protein were incubated with glutathione-coupled Sepharose beads. The GST-tagged 58E2 fragments were pulled down along with the protein associated with them. The precipitates were subjected to Western blot analysis with an anti-His antibody to detect whether the His–58E7 protein associated with a GST–58E2 fragment or not. As shown in Fig. 1(a), the His-tagged 58E7 was pulled down together with HDCT2, but not NT, suggesting that HPV-58E2 binds to 58E7 at the C-terminal region, which includes part of the 58E2 hinge domain (HD, 225–281 aa) and the C-terminal domain (CT, 282–358 aa).

58E2 protein binds 58E7 protein in a type-specific manner

A co-immunoprecipitation (co-IP) assay was also performed to confirm the interaction between 58E2 and 58E7. The vector for the full-length 58E2 fused with Myc tag (pCMV-3Tag-2A-58E2) was introduced into 293T cells fused with GST and 58E7 protein fused with 6xHis tag were used in a GST pull-down assay as described in Methods. The pull-down proteins were subjected to Western blot analysis (WB) with an anti-His antibody (lower panel). Input purified proteins (2 %) were analysed by WB, employing anti-GST (upper panel) and anti-His (middle panel) antibodies for the detection of GST–58E2 mutants and His–58E7, respectively. (b) 293T cell monolayers were co-transfected with Flag-tagged E7-expressing plasmids (pCMV-3Tag-1A-16E7/58E7) and Myc-tagged E2-expressing plasmids (pCMV-3Tag-2A-16E2/58E2). Twenty-four hours post-transfection, whole-cell extracts (WCE) were prepared from the transfected cells, and 4 % of the WCE was used to detect the expression of Myc–E2 and Flag–E7 proteins by WB, employing anti-Myc (upper panel) and anti-Flag (middle panel) antibodies, respectively. The remaining cell lysates were immunoprecipitated (IP) with a mouse-anti-Flag M2 affinity gel and the immunoprecipitates were resolved by SDS-PAGE and subjected to WB analysis with a rabbit anti-Myc antibody to detect the presence of E2 in the pellets (lower panel).
with Flag-tagged 58E7 expression vector (pCMV-3Tag-1A-58E7). At 24 h post-transfection, cells were lysed and subjected to an immunoprecipitation with a mouse anti-Flag antibody. Myc–58E2 was detected in the immunoprecipitate with a rabbit anti-Myc-tag antibody, indicating that 58E2 and 58E7 associate physically in 293T cells (Fig. 1b). Proteins 16E2 and 16E7 were included as a positive control, as their interaction has been identified before (Gammoh et al., 2006). Interestingly, no interaction was detected when 16E2 was co-expressed with 58E7. Similarly, 58E2 could not interact with 16E7 either, indicating that the interaction of E2 and E7 appears only to occur within an HPV type. Thus, we conclude that the interaction between E2 and E7 is HPV type-specific.

In addition, the E2 protein was also reported to interact with E6 of HPV-16 and -18 (16E6 and 18E6) in vitro. However, the E2 and E6 interaction was not detected in the low-risk HPV-11 (Grm et al., 2005). In our study, the interaction between 58E2 and HPV-58 E6 (58E6) was also examined. 293T cells were co-transfected with Myc–58E2 and Flag-58E6 expression vectors and a co-IP assay was performed as above. We failed to detect an interaction between 58E2 and 58E6, as well as 16E2 and 16E6 (data not shown).

**HD of 58E2 is responsible for binding 58E7**

To characterize the interaction between 58E2 and 58E7 further, a series of truncations of 58E2 were constructed, which produce Myc-tagged mutants of 58E2 (pCMV-3Tag-2A-58E2ms) (Fig. 2a). These Myc-tagged 58E2 mutants were respectively introduced into 293T cells with Flag-tagged 58E7 expression vector (pCMV-3Tag-1A-58E7) and the interaction between 58E2 mutants and 58E7 was examined by a co-IP assay as described above. The results demonstrate that the fragments of the HD202–254 or CT fused with different lengths of HD (HDCTs, including HDCT1, 2, 3) are able to bind 58E7, whereas the E2NT (1–201 aa) and E2CT (282–358 aa) alone cannot interact with 58E7, suggesting that HD of 58E2, especially the sequence between aa 244 and 254, is responsible for the association with 58E7 (Fig. 2b).

![Fig. 2. Mapping the domains of 58E2 binding to 58E7.](http://vir.sgmjournals.org)
The HD of HPV E2 is a flexible linker between the transactivation domain (the NT domain) and the DNA-binding domain (the CT domain). Among the three domains, the HD is the least conserved domain and shows very limited similarity among different types of HPV. The identity of HD is only 29%, in contrast to the identity of NT or CT, which is >50%. Its function is still not clear. The low similarity of the domain that is involved in the interaction with E7 proteins explains the type specificity of the E2 and E7 interaction.

58E2 protein inhibits 58E7-induced pRb degradation

Next, we investigated the biological significance of the interaction of 58E2 with 58E7. It has been demonstrated that 58E7 downregulates the cellular tumour suppressor pRb in primary human keratinocytes (PHKs) (Zhang et al., 2010). Furthermore, in HPV-16, the E2 protein has been found to inhibit 16E7-mediated pRb degradation (Gammoh et al., 2009). Therefore, we asked whether the 58E2–58E7 interaction represses 58E7-induced pRb degradation. Two lines of experiment were performed to address this question. First, we examined the effects of 58E2 on the steady-state levels of pRb in 58E7-expressing cells. HaCat and NIH3T3 cell lines were transiently co-transfected with the plasmids expressing the 58E2 or 58E2 truncations and 58E7. Twenty-four hours post-transfection, cycloheximide (CHX) was added to block new protein synthesis, ruling out any influence of altered protein synthesis on the pRb levels. The levels of pRb in a time-course of CHX treatment were analysed by Western blotting. The data obtained from HaCat cells showed that 58E7 was able to induce pRb degradation, whereas a mutant of 58E7 in which the pRb-binding motif (58E7A22–26) was deleted did not affect the pRb level (Fig. 3f, g). The presence of 58E2 protected pRb from 58E7-induced degradation (Fig. 3h). The same trend was seen in 16E2- and 16E7-transfected cells (Fig. 3b, c). The truncated fragments of 58E2, HDCTs and HD225 that bind 58E7 also exhibited the inhibition of the 58E7-induced degradation of pRb (Fig. 3j–m). However, the fragments of E2NT and E2CT that are not able to bind 58E7 failed to prevent the degradation of pRb (Fig. 3i, n). Taken together, our results indicate that the 58E2 activity in inhibition of 58E7-induced pRb degradation is dependent on the interaction with 58E7. As expected, the inhibition of pRb degradation was not observed when E2 and E7 were from different HPV types, as 16E2 could not rescue pRb from 58E7-induced degradation; neither could 58E2 rescue pRb from 16E7-induced degradation (Fig. 3d, e). Overall, 58E2 was demonstrated to target 58E7 and block 58E7-mediated pRb degradation. Similar results were seen in NIH3T3 cells (data not shown). It is suggested that the HD region of 58E2 is responsible for binding to 58E7 and affecting 58E7 function.

Secondly, we investigated whether 58E2 blocks 58E7-induced pRb degradation by competing with pRb to bind 58E7. In HPV-16, the domain of E7 that is responsible for interaction with pRb has been mapped to the pocket protein-binding motif LXCXE located at aa 22–26 in E7 (Münger et al., 1989). Sequences of the E7 protein are highly conserved in all HPV types, with 60% identity between 16E7 and 58E7. Furthermore, the region of aa 22–26 of 58E7 exhibits the same pRb-binding motif as 16E7. Thus, the mutant that omits aa 22–26 (58E7A22–26) is expected to abolish the pRb-binding activity of 58E7. This has been proven to be true by a co-IP analysis with 58E2 and 58E7 or 58E7A22–26 in HaCat cells (Fig. 4, bottom panel). We conclude that the region of aa 22–26 of 58E7 is responsible for binding pRb. However, 58E7A22–26 was found still to be able to bind 58E2 as effectively as the wild-type (WT) 58E7 (Fig. 4, lanes 2 and 3). This result suggests that it is unlikely that 58E2 inhibits 58E7-mediated pRb degradation by competing with pRb for binding to 58E7. Furthermore, in the co-IP experiment, we also observed that the presence of 58E2 even enhances the binding of pRb to 58E7 (or the 58E7–E2 complex) in comparison with the cells in the absence of WT 58E7 (which expressed the NT fragment that does not interact with 58E7) (Fig. 4, lowest panel, lanes 2 and 3). In other words, pRb appears to bind the E2–E7 complex better than E7 alone. Taken together, although 58E2 interacts with 58E7 and inhibits 58E7-induced pRb degradation, it does not block 58E7 binding to pRb, but appears to involve another mechanism to protect pRb from being degraded by 58E7.

58E2 protein blocks 58E7-induced cell proliferation

The pRb protein acts as a transcriptional repressor by targeting the E2F transcription factors, whose functions are required for G1- to S-phase cell-cycle transition. pRb is frequently inactivated by the HPV E7 protein in cervical cancer (Fiedler et al., 2004). It was suggested that 58E7 abrogates the G1–S checkpoint, increases the number of cells in the S phase and promotes the proliferation of PHK cells (Zhang et al., 2010). Given that 58E2 binds 58E7 and blocks E7-mediated pRb degradation efficiently, we hypothesized that 58E2 may inhibit cell proliferation induced by 58E7. To determine whether 58E2 can adversely affect the transforming function of 58E7, HaCat and NIH3T3 cells expressing 58E7 or 58E7 plus 58E2 were assayed for cell proliferation by colony-formation assay. Cells co-transfected with empty vectors (1A + 2A) served as a blank control, whilst 16E7-transfected and ‘16E7 + 16E2’-co-transfected cells served as a pair of positive controls. As shown in Fig. 5, both 16E7 and 58E7 could promote cell proliferation in HaCat cells and the numbers of cell colonies were significantly higher than those of control cells that were transfected with the empty vector (P<0.05). However, the numbers of cell colonies were reduced significantly to a level close to the control when the cells were co-transfected with 58E2 and 58E7, as well as with 16E2 and 16E7. The reduction of the colonies was also observed in the cells where 58E7 was co-transfected with 58E2.
**Fig. 3.** 58E2 protects pRb from 58E7-induced degradation. HaCat cells were transiently co-transfected with the expression vectors of HPV E7 and HPV E2 proteins or 58E2 truncation mutants as indicated. 1A and 2A represent the empty vector pCMV-3tag-1A and pCMV-3tag-2A, respectively. Twenty-four hours post-transfection, protein biosynthesis was blocked by addition of 100 μg CHX ml⁻¹ and cells were harvested and lysed at the indicated time points (0, 3, 6 h) after adding CHX. The equal amount of cell lysates protein was subjected to SDS-PAGE. The steady-state levels of pRb were determined by Western blot analysis (top panels; results shown are one of three independent experiments), and β-actin served as a loading control (lower panels). The densities of the pRb bands were normalized by comparison with the respective β-actin bands, and then the relative pRb values were expressed as a percentage where the value of 0 h CHX treatment in the same group corresponded to 100 %. Results shown are means ± SD of three experiments. *P<0.05 (Student’s t-test), pRb levels in CHX-treated cells for 0 h versus 6 h.
58E2 truncations, HDCTs and HD170–254, whereas no significant reductions were detected in cells where 58E7 was co-transfected with 58E2 mutants NT or CT (Fig. 5). Similar results were obtained in NIH3T3 cells (data not shown). The results indicated that the interaction between 58E2HD and 58E7 is necessary for 58E2 to block 58E7-associated cell proliferation.

**DISCUSSION**

HPV E2 is a multifunctional protein that plays very important roles in the virus life cycle, including regulating HPV genome replication, suppressing transcription of E6/E7 genes, modulating the host cell cycle and tethering viral genomic DNA to the mitotic chromosome (Breiding et al., 1997; Demeret et al., 1994; Steger et al., 1995; Tan et al., 1994; You et al., 2004). Banks and colleagues reported that HPV-16 E2 regulates 16E6 and 16E7 through direct protein interaction (Grm et al., 2005; Gammoh et al., 2006). In the current study, we found that 58E2 is also able to interact with 58E7 and negatively regulate its activity, but failed to bind 58E6.

Interestingly, the E2 and E7 interaction is found to only occur within the same type of HPV, as E2 of one type is unable to target E7 of other types, suggesting that the interaction is virus type-specific. The domain in 58E2 responsible for the interaction with 58E7 has been mapped to the HD. Among the different domains in HPV E2 proteins, namely the transactivation domain (NT domain), the DNA-binding domain (CT domain) and HD, the HD is highly variable in sequence and in length. Little has been known about its function. The HD had been long believed to be just a flexible linker between the NT and the CT. Although the HD is poorly conserved, Saitoh et al. (2008) found that the interaction of the E2 HD with E7 in HPV-1a, HPV-11 and HPV-16 can be blocked by podophyllotoxin, a compound able to bind the HD of E2, indicating that the E2 HD function in binding to the E7 protein could be conserved among different HPV types. There is evidence suggesting the importance of this domain for E2 function. For example, phosphorylation of serine residues in the HD of E2 of bovine papillomavirus type 1 is found to be necessary for the regulation of viral DNA replication (Lusky & Fontane, 1991; McBride & Howley, 1991); the linker is also critical for the regulatory functions of HPV-11 E2 during mRNA transcription and viral DNA replication (Zou et al., 2000). Furthermore, a dominant functional nuclear-localization signal was present in the HD of the HPV-11 E2 protein (Blachon et al., 2005), but absent from the E2 proteins of HPV-18 and HPV-16. The HD of 16E2 is probably located from aa 210 to 290 (Gauthier et al., 1991). The 58E2 HD is predicted by DNASTAR software to be between aa 202 and 281. The interaction of 16E2 and 16E7 was mapped to the region between aa 202 and 249 in HD (Gammoh et al., 2006). Our result indicates that the region between aa 244 and 254 in the 58E2 HD is responsible for interacting with 58E7. The variability and lack of conservation of the HD between different HPV types provides an explanation for the type specificity of the E2–E7 interaction.

HPV E6 and E7 are major oncoproteins of HPV. It was shown that expressing E7 alone in transgenic mice is sufficient to induce invasive cervical cancer (Riley et al., 2003). The E7 proteins from high-risk HPV types bind and degrade the members of the pocket protein family: pRb, p107 and p130 (Boyer et al., 1996; Dyson et al., 1992; Gonzalez et al., 2001). This results in the release of E2F proteins from the pRb–E2F complex, thus activating the transcription of genes required for S phase transition (Dyson et al., 1989; Giarré et al., 2001). The binding of E2 and E7 is therefore expected to reduce the levels of active E2F, leading to cell-cycle arrest. It has been demonstrated that the 58E7 protein downregulates pRb and p130 (Zhang et al., 2010). Our observation that 58E2 inhibited 58E7-induced pRb degradation provides evidence for a transcription-independent function of 58E2 to counteract the activity of the viral oncoprotein. Our result agrees with
previously published data showing the similar ability of 16E2 (Gammoh et al., 2009). Howley and colleagues previously reported that mutations in the E2-binding sites in the promoter for E6 and E7 expression partially relieved the negative effect of E2 on HPV-16-induced immortalization of PHKs, which was not as efficient as the E2-mutant virus, implicating an additional mechanism in the E2 repression of viral immortalization functions (Romanczuk & Howley, 1992). Our results proved their prediction. In addition, our results also explain the potential mechanism that E2NT alone only induces apoptosis, but not G1 arrest in HPV-positive cell lines (Demeret et al., 2003).

The mechanism underlying 58E2 inhibiting 58E7-induced pRb degradation and blocking 58E7-associated cell proliferation is still elusive. Our results have ruled out the possibility of 58E2 competing physically with pRb for binding to 58E7. We are continuing to investigate other possible models to reveal the mechanism of 58E2 counteracting 58E7-associated tumorigenesis potential. A thorough understanding of the mechanism is vital to comprehend HPV tumorigenicity and further investigation is definitely warranted.

Epidemiological studies indicated that HPV-58 is more prevalent in LSILs (6.8 %) and HSILs (10.7 %) than SCC (3.1 %) (Chan et al., 2009; Chao et al., 2011; Onuki et al., 2009), whereas the reverse is true for HPV-16 [LSILs (9.6 %), HSILs (24.1 %), SCC (40.5 %)] (Clifford et al., 2003; Onuki et al., 2009). The mechanism for the difference is not well understood. However, a molecular epidemiological study found that the median E6 loads of HPV-58

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**Fig. 5.** Inhibitory effects of 58E2 on 58E7-induced cell colony formation. The colony-formation assay was performed as described in Methods. Briefly, HaCat cells were co-transfected as indicated. Cells co-transfected with empty vectors ‘1A + 2A’ served as a blank control, while the 16E7-transfected and ‘16E7 + 16E2’ co-transfected cells served as a pair of positive controls. At 48 h post-transfection, 1×10⁵ cells of each set were resuspended and cultured in six-well plates, each well making two duplicates. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10 % FBS and 500 μg G418 ml⁻¹. (a) After 14 days, the colonies were fixed with 3.7 % paraformaldehyde and stained by crystal violet. (b) The numbers in the graph are the mean ± SD numbers of colonies from three independent experiments. *Values significantly different at P<0.05 (Student’s t-test or ANOVA test).
among women with LSILs and HSILs were not found to be significantly different, while the median E6 load of HPV-16 in women with HSILs was higher than among those with LSILs (Ho et al., 2006). One explanation for this observation might be that 58E2 is present in HPV-58-associated HSILs more frequently than 16E2 in HPV-16-associated lesions. This hypothesis is supported by the finding that only a small proportion of the integrated HPV-58 genome exhibited disruption of the whole E2 gene (Chan et al., 2007). Thus, the long period of whole or partially functional 58E2 existing in LSILs and HSILs might delay the progression of the lesions from HSIL to SCC. As a matter of fact, it raises a possibility that E2 may be able to serve as a therapeutic agent for cervical cancer. Since the E2 protein is a multifunctional transcription factor, the full-length E2 may not be appropriate for use in therapy, but the E2HD fragment, which is sufficient to interact with and inhibits the E7 protein, could be a potential candidate for a cervical cancer therapeutic agent. Given that the E2–E7 interaction is type-specific, development of such a therapeutic agent for cervical cancer is dependent on better understanding of the function of the HD in a variety of HPV types.

METHODS

Cells and E. coli strain. HaCat, NIH3T3 and human embryonic kidney (HEK) 293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% FBS (Gibco), 100 U penicillin ml\(^{-1}\) and 100 µg streptomycin ml\(^{-1}\). E. coli strains DH5α and BL-21 (DE3) were cultured at 37°C in lysogeny broth medium (Oxoid) and transformed as described previously (Sambrook et al., 1989).

Plasmids. For prokaryotic expression, the cDNAs of 58E7, 58E2 and the truncation mutants of 58E2 were amplified from the HPV-58 genome (presented by Dr T. Matsukura, National Institute of Health, Japan) by PCR employing specific primers (Table S1, available in JGV Online). PCR products were subsequently cloned into the prokaryotic expression vector with a 6 × His tag (pET21d) or glutathione S-transferase (GST) tag (pGEX-4T-1). The prokaryotic expression vectors were kindly provided by Dr Lichuan Gu (School of Life Science, Shandong University).

For eukaryotic expression, the genes of 16E2, 58E2 and the truncation mutants of 58E2 were cloned into pCMV-3tag-2A, which allows expression of 3 × Myc-tagged proteins. The E7 genes of HPV-16 and HPV-58 were cloned into pCMV-3tag-1A, which allows expression of 3 × Flag-tagged proteins. The 58E7A22–26 mutant was derived from pCMV-3tag-1A-58E7 by using a QuickChange II Site-Directed Mutagenesis kit (Agilent Technologies), which deletes aa 22–26 of the 58E7 gene.

Antibodies and Western blot analysis. Mouse anti-Flag (M2) antibody was purchased from Sigma, and rabbit anti-Myc-tag, rabbit anti-pRb and mouse anti-β-actin antibodies were all from Cell Signaling Technology. All antibodies were diluted at 1:1000 in blocking buffer (5% fat-free milk in PBS with 0.05% Tween-20).

Western blot analysis was performed as described before (Rozen et al., 2006). Briefly, cells were lysed in lysis buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 30 mM NaF, 5 mM EDTA, 10% (w/v) glycerol, 1 mM PMSF, 1% Nonidet P-40, 1 mM sodium orthovanadate) supplemented with protease inhibitor cocktail (Roche) for 30 min at 4°C, and then centrifuged at 20 000 g for 10 min at 4°C to remove the cell debris in a pellet. The supernatants were mixed with 4 × LDS sample buffer (Invitrogen) for SDS-PAGE. After being boiled for 10 min, proteins were separated on an SDS-PAGE 4–12% Bistris gel (Invitrogen) and transferred onto a nitrocellulose membrane (GE Healthcare Life Sciences). The membranes were blocked in blocking buffer for 1 h at room temperature, and subsequently incubated with the primary antibodies overnight at 4°C. They were then washed with PBST (0.05% Tween-20 in PBS) three times for 10 min, and incubated with the secondary antibodies conjugated to HRP (Pierce) for 1 h at room temperature. After the three washes, an enhanced chemiluminescence-based system (Pierce) was employed for detecting the proteins.

GST pull-down assay. GST fusion proteins (50 µg) were incubated with bacterially expressed and purified 6 × His-tagged 58E7 protein (50 µg) at 4°C for 2 h in 500 µl protein-binding buffer [50 mM Tris/HCl pH 8.0, 300 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 5 mM 2-mercaptoethanol (2-ME), 1 mM PMSF]. After incubation, 40 µl (packed volume) of glutathione–Sepharose 4B (GSH Sepharose; Pharmacia) was added, and the mixture was agitated at 4°C for 2 h. Then, the Sepharose beads were collected and washed five times with 1 ml protein-binding buffer with rotation at 4°C for 10 min each time. Finally, the proteins binding onto the beads were eluted by 1.5 × LDS sample buffer containing 2% 2-ME, and then the proteins were detected by SDS-PAGE and Western blot analysis.

Co-IP. Appropriate plasmids encoding Flag-tagged E7 and Myc-tagged E2 proteins were co-transfected into HEK 293T or HaCat cells by the calcium phosphate transfection method or Lipofectamine 2000 (Invitrogen), respectively. At 24 h post-transfection, whole-cell extracts were prepared by directly lysing cells from 100 mm plates in 0.5 ml lysis buffer A [50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1% Triton X-100 and protease inhibitor cocktail (Roche)]. On incubation ice for 30 min, the cells were scraped from the plates and the cell lysates were cleared by centrifugation at 20 000 g for 10 min at 4°C. The supernatants were incubated with 25 µl anti-Flag M2 affinity gel (Sigma) overnight on a rotating wheel at 4°C. The affinity gel was collected and washed four times with 500 µl lysis buffer A for 10 min each time. After washing, the affinity gel was resuspended in 1.5 × LDS sample buffer containing 2% 2-ME, and boiled for 10 min. The eluted proteins were analysed by Western blotting with the rabbit anti-Myc-tag or anti-pRb antibody.

Determination of steady-state level of pRb. HaCat and NIH3T3 cells were transfected with various plasmids by Lipofectamine 2000. At 24 h post-transfection, cells were treated with 100 µg cycloheximide ml\(^{-1}\) (CHX; Sigma) for 0, 3 and 6 h, respectively. Cells were harvested and suspended in lysis buffer B (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 30 mM NaF, 5 mM EDTA, 1 mM Na$_2$VO$_4$, 40 mM alpha-Glycero-phospho$\Delta$m, 10% glycerol, 1% Nonidet P-40, 1 mM PMSF and protease inhibitor cocktail), and incubated at 4°C for 30 min, and then the cell lysates were cleared by centrifugation at 20 000 g for 10 min at 4°C. The protein concentrations of the lysates were measured by Bradford assay kit (Bio-Rad) following the manufacturer’s instructions, and then equal amounts of proteins were loaded and subjected to SDS-PAGE and Western blot analysis. The Western blot results were photographed, and the density of the pRb bands was normalized by comparison with that of the respective -actin bands. The normalized values of pRb levels in each group were divided by the value of 0 h in the same group to obtain the relative values of pRb levels.

Colony-formation assay. HaCat and NIH3T3 cells were co-transfected with flag-tagged E7 and Myc-tagged E2 expression vectors by Lipofectamine 2000. At 48 h post-transfection, 1 × 10$^5$ cells of each
set were resuspended in 2 ml DMEM containing 10% FBS and 500 μg G418 ml⁻¹ (Cellgro), and cultured in six-well plates. Each well made two duplicates. The medium was changed every 3 days. After 14 days, the colonies were fixed with 3.7% paraformaldehyde (Sigma-Aldrich) for 10 min and stained with 0.005% crystal violet (Sigma-Aldrich) for 10–15 min, washed with tap water, then photographed. The number of colonies in each well was counted.

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