Human Papillomavirus Type 16 E7 Binds to E2F1 and Activates E2F1-driven Transcription in a Retinoblastoma Protein-independent Manner*

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The human papillomavirus (HPV) E7 oncoprotein can immortalize primary human cells and induce tumor formation. These properties of E7 depend on its ability to inhibit the activity of retinoblastoma protein (pRB), which in turn affects E2F function. E2F proteins control the expression of genes involved in differentiation, development, cell proliferation, and apoptosis. By using genetic and biochemical approaches, the present study shows that E7 binds to E2F1 in vitro and in vivo and that both proteins co-localize in the nucleolus. Importantly, the binding of the high risk group HPV E7 to E2F1 is tighter than the binding of the low risk group HPV E7 to E2F1. Although E7 of the high risk group HPVs activates E2F1-dependent transcription strongly in C33A or 293T cells, E7 of the low risk group HPVs activates transcription only weakly. By using electrophoretic mobility shift assay, we also showed that E7 binds to E2F1-DNA complexes. Furthermore, we show that these activities of E7 are independent of pRB by using E7 and E2F1 mutants that cannot bind to pRB. Taken together, these data suggest that E7 contributes to the deregulation of pRB-dependent E2F1 repression and to the further activation of E2F1 independently of pRB.

Human papillomaviruses (HPVs)1 are small DNA viruses that are the etiological agents of cervical cancer, and more than 100 different genotypes of HPV have been isolated (1). HPV can be divided into two subtypes as follows: low risk group HPVs such as HPV-6b and -11 that are associated with benign proliferative lesions, and high risk group HPVs such as HPV-16 and -18 that are associated with malignant tumors (2, 3). In cervical cancers, HPV E6 and E7 are major transforming proteins. The E7 protein of the high risk group HPVs cooperates with the E6 protein to immortalize human keratinocytes (3–5). E7 protein of the high risk group HPV binds to pRB with higher affinity than does E7 protein of the low risk group HPV, and the binding affinity of pRB to E7 correlates with the transforming potential of E7 (3). Through interaction with the retinoblastoma (RB) family of proteins such as pRB, p107, and p130, E7 disrupts the pRB-E2F complex and deregulates the repressive function of pRB in cell cycle progression (2, 6–12). Previous data also showed that HPV-16 E7 leads to degradation of pRB and is required for the productive stage of the viral life cycle (13–15). Recently, many proteins have been reported to bind to HPV-16 E7, and this interaction is related to the oncogenic potential of E7 (16–18).

E2F proteins play an important role in the regulation of cell cycle progression and entry into S-phase. Furthermore, E2Fs are involved in the regulation of transcription of several genes necessary for differentiation, development, proliferation, and apoptosis (19, 20). E2F-binding sites are found in the promoters of genes required for nucleotide synthesis (dihydrofolate reductase and thymidine kinase), DNA replication (DNA polymerase α and cdc6), and cell cycle progression (cyclin E, cdc2, and c-Myc) (21). E2F activity is regulated by interactions with RB family members (22). When E2F is associated with RB family members, the pRB-E2F complex functions as a transcriptional repressor, whereas free E2F activates transcription (23–26). During G1 to S-phase progression, the inhibitory activity of pRB is disrupted by its phosphorylation by cyclin-dependent kinases (27, 28).

There are six members of the E2F family, E2F1–E2F6, that can be sub-divided into three classes (21, 23, 25). E2F1–3 bind to pRB, and the ectopic expression of E2F1–3 is sufficient to drive cells into S-phase. E2F4–5 bind to all three RB family members, but these E2Fs are unable to induce S-phase entry in quiescent cells. E2F6 is a transcriptional repressor, but its physiological functions are not well characterized. Recently it was reported that E2F6 is a component of the mammalian Bmi-1-containing polycomb complex (29).

E2F1 has been implicated as an oncogene because its over-expression can drive quiescent cultured cells through G1 into S-phase of the cell cycle, ultimately leading to apoptosis or neoplastic transformation (30). Increased E2F1 activity induces skin tumors in mice heterozygous or nullizygous for p53 (31). Previous data imply that E7 transactivation involves interaction with E2F proteins (32). It was reported that the E2F-cyclin A complex associates with the E7 protein in extracts of Caski cells, which express high levels of HPV-16 E7 protein. This E7-bound E2F-cyclin A complex might be an important intermediate in E7-mediated transformation (33). In transgenic mice the ability of HPV-16 E7 to alter the fate of fiber cells is partially dependent on E2F1 (34). These data provide genetic and biochemical evidence that E2F1 is a mediator of E7 in vivo.
The present study is the first to report a functional interaction between E7 and E2F1. We show that E7 and E2F1 directly interact to activate E2F1-driven transcription. We also identify the binding domains of each protein. The binding affinity to E2F1 differs between E7 of the high risk group HPVs and E7 of the low risk group HPVs and correlates with a difference in activation of E2F1-driven transcription. Furthermore, we report that E7 activates E2F1-driven transcription even in the absence of pRB and that HPV-16 E7 forms a complex with E2F1-DNA complexes. These data suggest that HPV-16 E7 binding to E2F1 is an important event in cell transformation by HPV.

EXPERIMENTAL PROCEDURES

Plasmids—PcG8/SFLAG2E7F1 and PcG8/SFLAG2E7F1(Y411C) were gifts from Michael D. Cole (Princeton University). pEG2T/K-E2F1 and pCMV2E2F1 were gifts from William G. Kaelin (Dana-Farber Cancer Institute). pEGF4C1 16 E7 was a gift from E. Aubrey Thompson (University of Texas). pBKX-1 16 E7-1(409–437) and DHR-Gal4 reporter plasmid were gifts from Peggy J. Farnham (University of Wisconsin Medical School). HPV-16 E7 mutant (pLXS3) was a gift from Soo-Jong Um (Sejong University, Korea). HPV E7 genes and HPV-16 E7(1–39), HPV-16 E7(1–40)–(39), and HPV-16 E7-(40–98) were obtained as restriction fragments of EcoRI and SalI by polymerase chain reaction, using the appropriate primers, and cloned into pGEX4T-1 to make the glutathione-S-transferase (GST) fusion protein using bacterial expression vectors. HindIII-SalI restriction fragments of HPV-6b, -11, -16, and -18E7 were generated by PCR, and each was cloned into pFLAGCMV2 to make FLAG-tagged expression vectors of E7 protein. pEG200-16 E7 (BamHI/SalI, for His-tagged protein), pEGI 16 E7 (BamHI/NcoI, for GST-tagged expression vector), and pEGFP-C1 16 E7 (BglII/SalI, for green fluorescent protein (GFP)-tagged expression vector, CLONTECH) were generated by digesting pGEX4T-1 16 E7 with the appropriate restriction enzymes. pGEMX-1, pFLAGCMV2 16 E7 mutant, was obtained as a restriction fragment of EcoRI and SalI by PCR using the pLXS316 E7 mutant template. pCDNA3/E2F1, pGEX4T-1/E2F1, and pEG/E2F1 were cloned by inserting corresponding PCR fragments into the multicloning site of each mammalian expression vector. HindIII-XhoI restriction fragment of RB was generated by PCR and was cloned into pFLAGCMV2 (HindIII-SalI) to make FLAG-tagged expression vectors of RB. pGEX4T-1/1B (ABC pocket) was obtained as a restriction fragment of EcoRI and XhoI by PCR.

Cells, Transfection, and Reporter Assay—C33A (a pRB negative cell line) and 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. At 24 h before transfection, 3 × 10^6 cells were seeded into 6-cm dishes. Transfections were performed by the calcium phosphate method (35). The transfected plasmids were prepared by the Qiagen procedure (Qiagen, Hilden, Germany), and the total amount of DNA transfected was adjusted with the control plasmid DNA lacking the cDNA to be expressed. Equal amounts of cell lysates were employed for the detection of luciferase activity. In each transfection assay, an RSV-β-gal expression vector was co-transfected, and β-galactosidase activity was measured as an internal control for transfection efficiency.

Protein Purification and GST Pull-down Assays—GST and GST fusion proteins were expressed in bacteria and purified according to the manufacturer’s recommended protocol (Amersham Bioscience). His fusion proteins were expressed in bacteria and purified according to the manufacturer’s recommended protocol (Qiagen). GST fusion proteins were incubated with [35S]methionine-labeled proteins expressed in vitro translation (using T7 Quick TNT kit as described by the manufacturer (Promega, Madison, WI)). After 30 min of incubation at room temperature in binding buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol, and 0.5% Nonidet P-40), glutathione-Sepharose 4B beads (Amersham Bioscience) were added and incubated further for 40 min at room temperature. Incubated beads were washed four times with binding buffer, and bound proteins were analyzed by SDS-PAGE and autoradiography.

Immunoprecipitation and Immunoblotting—Cells expressing GST and GST-HPV-16 E7 with FLAG-E2F1 were lysed in EBC buffer (50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 200 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride) and incubated with 30 μl of a 1:1 suspension of glutathione-Sepharose or protein G resin (Amersham Bioscience) in EBC buffer for 4 h at 4 °C with rocking. The glutathione-bound or protein G resin-bound complexes were then washed three times with EBC buffer and boiled at 95 °C for 5 min in SDS sample buffer. Immunoblot analysis was carried out using anti-FLAG antibody (Sigma) or anti-GST monoclonal antibody. Cells were scraped into microfuge tubes, lysed in SDS sample buffer, and heated to 95 °C for 5 min, and 10–20 μg of protein sample was separated on SDS-PAGE and electroblotted onto nitrocellulose membranes. The blots were blocked and incubated sequentially with primary antibodies and horseradish peroxidase-coupled secondary antibodies. Proteins were detected by chemiluminescence with ECL substrate (Amersham Bioscience).
Immunofluorescence—GFP-fused HPV-16 E7 (1 μg) and expression vectors containing FLAG-tagged E2F1 were transfected into 293T cells (1 μg). Cells were fixed and immunostained for 24 h after transfection. FLAG-tagged E2F1 was detected using a rhodamine-conjugated secondary antibody. Immunofluorescence was detected using a confocal laser scanning microscope (LSM510, Carl Zeiss, Jena, Germany).

Electrophoretic Mobility Shift Assay—Assays were performed using purified proteins described above and a 32P-labeled double-stranded DNA oligonucleotide probe representing the E2F1-binding site, 5'-ATTTAAGTTTCGCGCCCTTTCTCA-3'. Probe containing the E2F1-binding site was labeled in the presence of [γ-32P]ATP (Amersham Bioscience) and T4 polynucleotide kinase. Labeled nucleotides were incubated at room temperature with GST-E2F1 protein for 30 min. For the supershift assay, His-tagged HPV-16 E7 was added to the oligonucleotide complex after a 10-min binding reaction, and the mixture was incubated for an additional 20 min at room temperature. The protein-DNA complexes were resolved on an 8% acrylamide gel in 0.25 TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA). The gels were dried and visualized by autoradiography.

RESULTS

E2F1 Binds to HPV-16 E7—Although the ability of E7 to transform cells depends on its binding to pRB, binding alone is not sufficient for transformation (36). Previous studies (33, 34, 37) implied that the inhibition of pRB activity and consequent stimulation of E2F-dependent transcription by E7 was dependent on E2F1 protein. We performed in vitro binding assays to identify a possible interaction between HPV-16 E7 protein and E2F1 protein. E2F1 was translated in vitro using the rabbit reticulocyte system and labeled with [35S]methionine. Labeled lysates were incubated with either purified GST, GST-HPV-16 E7, or GST-RB fusion protein. The data presented in Fig. 1A showed that GST-HPV-16 E7 binds to E2F1. The control incubations show that GST alone does not bind to E2F1, whereas GST-RB tightly binds E2F1 in vitro, as expected. No proteins bind to in vitro translated luciferase protein (negative control).

We next tested whether HPV-16 E7 binds to E2F1 in vivo. A plasmid expressing GST-HPV-16 E7 protein was transiently co-transfected with a FLAG-tagged E2F1 expression plasmid into 293T cells. We used GST alone, with or without a FLAG-tagged E2F1 expression vector, as a negative control. Selective precipitation from the cell lysate of GST protein using GST resin or FLAG-tagged protein using FLAG antibody-protein G resin showed the co-precipitation of FLAG-E2F1 protein and GST-HPV-16 E7 protein (Fig. 1, B and C). These data confirm that HPV-16 E7 protein and E2F1 indeed associate in mammalian cells.

E2F1 consists of many domains, such as the cyclin A binding domain, DNA binding domain, dimerization domain, and transactivation domain (Fig. 2A). To identify the regions of E2F1 that are required for binding to HPV-16 E7, in vitro binding assays were performed by incubating GST fusion proteins encoding various domains of E2F1 with His-tagged HPV-16 E7 protein. Eight GST-E2F1 deletion mutants and GST protein (negative control) were incubated with purified His-tagged HPV-16 E7 protein, and binding was detected using anti-His monoclonal antibody. The data presented in Fig. 2B show that the wild type E2F1, GST-E2F1-(1–38), GST-E2F1-(191–368), and GST-E2F1-(284–437) strongly bind to E7,
binding also differs between the high and low risk groups of HPV E7, we performed in vitro binding assays using in vitro translated E2F1 and various types of GST-HPV E7 proteins. Labeled lysates were incubated with either purified GST, GST-HPV-6b E7, GST-HPV-11 E7, GST-HPV-16 E7, or GST-HPV-18 E7 fusion proteins. We found that although E7 proteins of the high risk group HPV's bound to E2F1, E7 of the low risk group HPV's did not bind as efficiently as to E2F1 (Fig. 3A). In low salt buffer conditions (100 mM NaCl), E7 of the low risk group bound to E2F1 but with a lower affinity than E7 of the high risk group.

The apparent differences in binding affinity were further investigated by comparing the transactivation activity of E7 using a synthetic E2F1-dependent promoter containing two E2F1-binding sites and a thymidine kinase core promoter fused to the luciferase gene (p6Ex2TK-E2F1). C33A cells were co-transfected with plasmids coding for various HPV-E7 subtypes, E2F1 and the p6Ex2TK-E2F1 reporter constructs. We found that E2F2-dependent transcription was greater in the cells expressing E7 protein of the high risk group HPV's (1.5–2.5 times control) compared with cells expressing E7 of the low risk group HPV's (1–1.6 times control). We showed that expression of E2F1 was not changed by E7 using Western blotting (Fig. 3B). Examination of E7 protein expression by Western blotting (Fig. 3C) revealed that high risk group HPV E7 was weakly expressed compared with low risk group HPV E7 (even though E2F1-dependent promoter activity was more strongly stimulated by the high risk group E7). From these results we conclude that E7 of the high risk group more tightly binds to E2F1 and more strongly activates E2F1-dependent transcription, compared with E7 of the low risk group HPV.

HPV-16 E7 Activates E2F1-driven Transcription in 293T and C33A Cells—To investigate further the functional significance of the E2F1 and HPV E7 interaction, we transfected 293T and C33A cells with E2F1 and E7 expression vectors and three different types of reporter plasmids. HPV-16 E7 transactivated the E2F1-driven transcription on the synthetic E2F1-dependent promoter in 293T cells and pRB-negative C33A cells (Fig. 4, A and B). We next tested whether HPV-16 E7 activates other E2F1-dependent reporter plasmids. It has been reported that the cyclin E promoter (pE) is an E2F-dependent promoter (39). The pE promoter was activated by HPV-16 E7 expression plasmid in a dose-dependent manner (Fig. 4C). When co-transfected with E7 and E2F1 expression plasmids, the pE promoter was further activated by HPV-16 E7 in a dose-dependent manner (Fig. 4D). We also tested synthetic E2F1-dependent promoter (E2FLuc) and observed similar results (data not shown). A previous report (40) showed that Gal4-E2F1 activates dihydrofolate reductase (DHFR)-Gal4 promoter in NIH3T3 cells. The DHFR-Gal4 reporter plasmid contains a Gal4-binding site in precise replacement of the E2F1-binding site in the DHFR promoter. To show that the physical interaction of HPV-16 E7 and E2F1 is important, C33A cells were transfected with HPV-16 E7 and Gal4-E2F1 (pM E2F1) or Gal4-E2F1-(409–437) reporter constructs. Gal4-E2F1-(409–437) contains only a small part of E2F1, but this domain alone is sufficient to activate DHFR-Gal4 reporter and interacts with CREB-binding protein (40). However, this domain could not bind to HPV-16 E7 (Fig. 2B). The data presented in Fig. 4E show that HPV-16 E7 activated the DHFR-Gal4 reporter in a dose-dependent manner, whereas Gal4 or HPV-16 E7 alone did not. HPV-16 E7 did not activate Gal4-E2F1-(409–437)-driven transcription (Fig. 4F). These results indicate that physical interaction between HPV-16 E7 and E2F1 is important for activating E2F1-dependent transcription.

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**A**

whereas GST-E2F1-(1–190), GST-E2F1-(1–120), GST-E2F1-(369–437), and GST-E2F1-(409–437) did not bind to E7. In low salt buffer conditions (100 mM NaCl), GST-E2F1-(369–437) bound to E7 but with a lower affinity (data not shown). These results indicate that the E7 binding region within E2F1 lies predominantly within amino acids 284–368 of E2F1.

By using E7 deletion mutants, we next identified the region of E7 that binds to E2F1. GST-E7, GST-E7-(1–39), or GST-E7-(40–98) was incubated with in vitro translated E2F1 protein. E2F1 bound to the C-terminal domain of HPV-16 E7 (Fig. 2C).

**E2F1 Binds More Strongly to High Risk Group E7 Than to Low Risk Group E7, and Its Binding Activity Relates to E2F1-driven Transcription**—HPV-E7 proteins of the low risk group associate with pRB with a lower affinity than HPV-E7 proteins of the high risk group (2, 3). To determine whether E2F1 binds more strongly to high risk group HPV-E7 than to low risk group HPV-E7, we performed in vitro binding assays using in vitro translated E2F1 and various types of GST-HPV E7 proteins. Labeled lysates were incubated with either purified GST, GST-HPV-6b E7, GST-HPV-11 E7, GST-HPV-16 E7, or GST-HPV-18 E7 fusion proteins. We found that although E7 proteins of the high risk group HPV's bound to E2F1, E7 of the low risk group HPV's did not bind as efficiently as to E2F1 (Fig. 3A). In low salt buffer conditions (100 mM NaCl), E7 of the low risk group bound to E2F1 but with a lower affinity than E7 of the high risk group.

**Figure 3**. Functional interaction between E2F1 and E7 of high risk (HPV-16 and HPV-18) and low risk (HPV-6b and HPV-11) group HPVs. A, binding of E2F1 and various HPV E7 proteins. In vitro translated and radiolabeled E2F1 proteins were incubated with purified GST or GST-HPV-6b, -11, -16, and -18 E7 proteins and immobilized on glutathione-Sepharose 4B beads in various salt concentrations. Bound proteins were separated on a 10% SDS-PAGE and analyzed by autoradiography. The relative photostimulated luminescence (PSL) unit was calculated by normalizing it to that of the background. B, E7 of different HPV types transactivates E2F1-driven transcription to different levels. Expression of E2F1 was confirmed by Western blot. C, expression level of HPV E7 (15% SDS-PAGE). C33A cells were transfected with 2 μg of each FLAG-tagged E7 expression vector, and cell lysates were immunoblotted using anti-FLAG antibody.

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**B**

**C**

**D**

**E**

**F**
**pRB-independent Manner**—We showed that HPV-16 E7 activates E2F1-dependent transcription in pRB-negative C33A cells. To confirm that HPV E7 can stimulate E2F1-dependent promoter activity in a pRB-independent manner, we performed experiments using HPV-16 E7(21–24), which does not contain the pRB-binding motif LXX(41, 42). First we showed that HPV-16 E7(21–24) can bind to E2F1 (Fig. 5A). Next, we transfected HPV-16 E7(21–24) and pE reporter plasmids into C33A cells. The data presented in Fig. 5B show that HPV-16 E7(21–24) activates the pE reporter in a dose-dependent manner. The level of HPV-16 E7(21–24) protein expression was similar to the wild type HPV-16 E7 (Fig. 5C). To determine whether E7 activates the E2F1-dependent promoter without affecting endogenous pRB or ectopically expressed pRB, we performed a transient transcription assay using an E2F1 mutant (pCps/FLAGE2F1-Y411C) which cannot bind to pRB but has transactivational activity (38, 43, 44). pCps/FLAGE2F1-Y411C was transfected with the p6Ex2TK-E2F1 reporter plasmid and the HPV-16 E7 expression plasmid into C33A cells. HPV-16 E7 was found to activate the synthetic E2F1-dependent reporter in a dose-dependent manner (Fig. 5D). Co-transfection of a pRB construct with the mutant E2F1 vector did not affect this p6Ex2TK-E2F1 reporter activity (Fig. 5E).

**HPV-16 E7 and E2F1 Co-localize in the Nucleus**—To confirm that E7 binds to E2F1 in vivo, we assessed whether HPV-16 E7 and E2F1 colocalize in 293T cells. HPV-16 E7 is a phosphoprotein that has been detected in the cytoplasm by immunoprecipitation and in the nucleus by immunofluorescence (45, 46), whereas E2F1 is a known nuclear protein (24, 47, 48). Under our conditions, GFP or FLAG-tagged HPV-16 E7 was seen mainly in the nucleus, whereas E2F1 was only observed in the nucleus. GFP showed a diffuse location pattern throughout the cell (Fig. 6A). To test whether E7 and E7(21–24) co-localized with E2F1 in mammalian cells, 293T cells were co-transfected with expression vectors for FLAG-tagged E2F1 in combination with GFP-tagged HPV-16 E7 or GFP-tagged HPV-16 E7(21–24). Examination of these transfected cells showed the presence of yellow color in the nucleus, indicative of co-localization of the two ectopically expressed proteins. This result is consistent with our finding that HPV-16 E7 associates with E2F1 for transactivation of E2F1-driven transcription.

**E7 Associates with an E2F1-DNA Complex**—E2F1 is a DNA-binding protein, and this binding is important for E2F1-dependent transcription (49, 50). Given HPV-16 E7 activated E2F1-dependent transcription independently of pRB (Figs. 4 and 5), we investigated whether HPV-16 E7 could associate with an E2F1-DNA complex. By using electrophoretic mobility shift assay, we showed that when labeled DNA probe containing an E2F1-binding sequence was incubated with GST-E2F1 in increasing amounts, the intensity of the band was increased (Fig. 7A, lanes 2–4), whereas the addition of a 30–150-fold excess of “cold” oligonucleotide (containing the E2F1-binding sequence) reduced the band intensity (lanes 5 and 6). The addition of His-16 E7 did not affect the migration of the DNA...
probe alone, but it did supershift the E2F1-DNA complex in a dose-dependent manner (Fig. 7, lanes 3–5, arrowhead C2). These results indicate that HPV-16 E7 binds to an E2F1-DNA complex and forms a ternary complex for activating E2F1-dependent transcription.

**DISCUSSION**

Different DNA tumor viruses produce distinct viral factors that can share common functions. For example, the HPV-16 E7 protein and the adenovirus E1A protein both activate the ras oncogene to transform primary rat kidney cells (51). They also activate adenovirus E2 promoter that contains E2F-binding sites, and this activity is mediated through interaction with cellular factors (52–56). HPV E7, SV40 large T antigen, and adenovirus E1A are well known viral factors that bind to pRB and deregulate its function (57, 58). However, studies of several HPV E7 mutants demonstrated that pRB destabilization was not sufficient to overcome cell cycle arrest of keratinocytes, suggesting other functions of E7 are also necessary for transformation of host cells (36). In this study, we showed that HPV-16 E7 protein interacted with E2F1 both in vitro and in vivo. We also found that E7 stimulated E2F1-driven transcription, which correlates with the ability of E7 to induce transformation or deregulate the cell cycle. The stimulatory effect of E7 on E2F1-driven transcription was not dependent upon pRB because E7 was able to stimulate E2F1-driven transcription in C33A cells (a pRB-negative cell line), and an E7 mutant that cannot bind to pRB was able to activate E2F1-dependent transcription in C33A cells. These results indicate that in addition to pRB, E2F1 is a functional target for HPV-16 E7.

Similarly, the present work showed that the binding affinity of E7 for E2F1 and the transactivational activity of E7 on E2F1-driven transcription differed according to the HPV types. In studies using a synthetic E2F1 promoter, we showed that E7 of the high risk group HPVs stimulates more strongly E2F1-driven transcription compared with E7 of the low risk group HPVs. We also tested other E2F-dependent promoters (cyclin E and adenovirus E2) and observed similar results (data not shown). These findings suggest that differences in the ability of E7 to both bind and transactivate E2F1 are related to the transformation potential of E7.

Interestingly, we found that an HPV-16 E7 mutant, 16E7(H900421–24), which cannot bind to pRB family members and is known to be transformation-deficient (41, 42), was able to bind to E2F1 and activate E2F1-driven transcription (Fig. 5B). These experiments were carried out in pRB-negative C33A
cells to exclude the involvement of pRB. We further confirmed that HPV-16 E7 can activate E2F1-dependent transcription in a pRB-independent manner by using the mutant E2F1(Y411C), which cannot bind to pRB but retains transactivation activity (36, 43, 44). In C33A cells the transcriptional activity of this E2F1 mutant was further stimulated by HPV-16 E7 in a dose-dependent manner (Fig. 5D).

Previous studies (60) showed that E7 expression leads to degradation of pRB and reduction of pRB levels (14, 15, 59). However, destabilization of pRB is not sufficient to transform host cells. Our data suggest that in addition to the E7-pRB interaction, an E7-E2F1 interaction also contributes to cellular transformation. We showed that E2F1 binds to the C-terminal half of E7 (residues 40–60), which contains a zinc finger region (Fig. 2C). Previous data showed that the oncogenic potential of E7 is severely reduced in the C terminus deletion mutant, and the C-terminal domain of E7 is involved in the E2F competition for pRB (17, 36, 37, 51, 61). E2F competition may be explained by E7-E2F1 binding. Helt et al. (60) suggested that the C terminus of E7 is involved in an additional activity required for abrogating keratinocyte G1/S control. Our results suggest that the C terminus of E7 also contributes to the deregulation of cell cycle control and strengthens the notion that several regulatory pathways must be subverted by E7 before host cells are transformed. Our findings are consistent with previous results from genetic (14, 41, 60, 62–64) and biochemical (8, 17, 33, 65, 66) experiments.

Overexpression of E2F1 induces apoptosis. A significant portion of apoptosis observed in RB−/− mice is eliminated when the mice are crossed with an E2F-1−/− background. In the absence of pRB, the resulting free E2F1 accumulates and triggers apoptosis (11, 16, 62, 67–70). We also observed that overexpression of E2F1 with E7 induced apoptosis more than E2F1 alone (data not shown). This phenomenon may be explained by the ability of E7 to increase the activity of E2F1.

We showed that E7 associates with E2F1-DNA complexes (Fig. 7). E2F1 binding to DNA is important for its role in activating transcription (49, 50), and the acetylation status of E7 is also important for DNA binding (71). E7 has a transactivation function similar to adenovirus E1A and can bind to other general transcription factors such as TATA-box binding protein and AP1 family members to contribute to transforming activity (51, 52, 54, 55). We speculate that the transactivation activity of E7 is important for stimulation of E2F-dependent transformation. Alternatively, we cannot rule out the possibility that E7 functions as a mediator to recruit a general co-activator such as CREB-binding protein and p300/CREB-binding protein-associated factor (pCAF).

In summary, we conclude that E7 interacts with E2F1. This functional interaction results in the activation of E2F1-driven transcription which contributes to deregulation of the cell cycle and induction of transformation.
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