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E2 Proteins from High- and Low-Risk Human Papillomavirus Types Differ in Their Ability To Bind p53 and Induce Apoptotic Cell Death

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The E2 proteins from oncogenic (high-risk) human papillomaviruses (HPVs) can induce apoptotic cell death in both HPV-transformed and non-HPV-transformed cells. Here we show that the E2 proteins from HPV type 6 (HPV6) and HPV11, two nononcogenic (low-risk) HPV types, fail to induce apoptosis. Unlike the high-risk HPV16 E2 protein, these low-risk E2 proteins fail to bind p53 and fail to induce p53-dependent transcription activation. Interestingly, neither the ability of p53 to activate transcription nor the ability of p53 to bind DNA, are required for HPV16 E2-induced apoptosis in non-HPV-transformed cells. However, mutations that reduce the binding of the HPV16 E2 protein to p53 inhibit E2-induced apoptosis in non-HPV-transformed cells. In contrast, the interaction between HPV16 E2 and p53 is not required for this E2 protein to induce apoptosis in HPV-transformed cells. Thus, our data suggest that this high-risk HPV E2 protein induces apoptosis via two pathways. One pathway involves the binding of E2 to p53 and can operate in both HPV-transformed and non-HPV-transformed cells. The second pathway requires the binding of E2 to the viral genome and can only operate in HPV-transformed cells.

Human papillomaviruses (HPVs) that infect the genital tract can be divided into two groups. High-risk (HR) or oncogenic viral types, such as HR-HPV type 16 (HR-HPV16) and HR-HPV18, are associated with cervical cancer and some other human tumors (7). In contrast, low-risk (LR) or nononcogenic types, such as LR-HPV6 and LR-HPV11, cause genital warts and are not associated with cancer. An appreciation of the differences between the HR- and LR-HPV types is central to an understanding of the origins of cervical cancer and other HPV-induced diseases. The E6 and E7 proteins from HR-HPV have long been recognized to be oncoproteins (33), and significant differences in the properties of E6 and E7 proteins from HR and LR viral types have been identified. For example, the E6 proteins from HR-HPV16 and HR-HPV18 bind to the p53 tumor suppressor protein, resulting in increased degradation of p53 (22, 41). Although the E6 proteins from LR-HPV6 and LR-HPV11 can also bind to p53, these interactions do not increase p53 degradation (6, 28, 41). The E7 proteins from HR- and LR-HPV types also show significant differences. Although the E7 proteins from HR-HPV16 and HR-HPV18 bind to the retinoblastoma tumor suppressor protein p105Rb in vitro, the E7 proteins from LR-HPV6 and LR-HPV11 bind this protein poorly (16, 34). Interesting though these differences in the E6 and E7 proteins are, it is important to note that the HR-HPV infections are relatively common and the presence of an HR-HPV type does not therefore always result in oncogenesis (36). Thus, although these differences in the E6 and E7 proteins from different HPV types are undoubtedly important in the establishment and/or progression of cervical cancer, they cannot in themselves completely explain why some HPV types are oncogenic while others are not.

In cervical warts the HPV genome exists as an extrachromosomal DNA circle. However, in cervical cancer cells HR-HPV DNA is often integrated into the host genome (13, 42, 46). Although the integrated genomes retain the E6 and E7 open reading frames (ORFs), the viral E2 ORF is often disrupted, resulting in loss of the E2 protein (2, 5). In other cases, the viral E1 ORF is disrupted (26). Since the E2 ORF lies downstream of the E1 ORF and since they are both under the control of a single promoter, disruption of the E1 ORF also results in the loss of E2 expression. Interestingly, cells containing integrated HR-HPV16 DNA have been shown to possess a selective growth advantage over cells containing nonintegrated HPV DNA (25). Furthermore, disruption of the E2 ORF has been shown to increase the immortalization capacity of HR-HPV16 (38). These observations suggest that the loss or inactivation of the E2 protein plays a role in cervical cancer.

The HPV E2 protein is required for efficient viral replication and is also thought to regulate viral gene expression (7). Disruption of the E2 ORF thus blocks viral replication and leads to the deregulation of viral gene expression. This loss of control over E6 and E7 expression has long been thought to contribute to oncogenesis. However, recent work suggests that the E2 proteins might also play a more direct role in the regulation of cell proliferation and cell death. The E2 proteins from several HR-HPV types have been shown to induce growth arrest and/or apoptosis (11, 40). The HR-HPV16 and HR-HPV18 E2 proteins can both induce growth arrest in HeLa cells, an HPV18-transformed cervical carcinoma cell line, by repressing transcription of the integrated E6 and E7

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genes (12, 14, 18, 23, 24, 35). The E2 proteins from HR-HPV types 16, 18, and 33 can also induce apoptotic cell death in some non-HPV-transformed cell lines (12, 15, 45). These data suggest that the E2 proteins can alter cell growth via their effects on the expression of E6 and/or E7 and via a separate pathway that does not depend on their ability to regulate these oncogenes. In support of this conclusion, the E2 proteins from HR-HPV16 and HR-HPV18 have been shown to interact with at least two proteins that can modulate cell growth and cell death: p53 (32) and CBP (29). p53 is a tumor suppressor protein that can bring about cell cycle arrest and/or apoptosis in response to a number of stimuli, including ionizing radiation, cell stress, or viral infection (4, 44). CBP is a member of a family of coactivator proteins that activate gene expression by virtue of their acetyltransferase activity and their ability to interact with transcription factors and components of the basal transcription machinery (37, 47). CBP is thought to be important in some forms of p53-dependent apoptosis (1).

Here we show that two LR-HPV E2 proteins fail to induce apoptosis in HPV-transformed cells and non-HPV-transformed cells. We show that the HR-HPV16 E2 protein induces apoptosis by at least two pathways. One pathway requires a direct protein-protein interaction with p53 and can function independently of the presence of the HPV genome. The second pathway requires the DNA binding activity of the HR-HPV16 E2 protein and the presence of the HPV genome.

MATERIALS AND METHODS

Cell culture and transfections. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS; GIBCO), 100 μg/ml streptomycin (Evans Medical Ltd.), and 60 μg/ml penicillin (Glaxo Laboratories Ltd.). HeLa, SiHa, CaSki, NIH 3T3, Saos-2, and MCF-7 cells were maintained in DMEM supplemented with 10% FBS, penicillin (10^5 units liter^{-1}), and streptomycin (100 mg liter^{-1}). 879 cells were maintained in DMEM supplemented with 5% FBS, insulin (5 μg ml^{-1}), hydrocortisone (10 mg μl^{-1}), penicillin (10^5 units liter^{-1}), and streptomycin (100 mg liter^{-1}). 808F and 873F cells were maintained in DMEM supplemented with 5% FBS, insulin (5 μg ml^{-1}), epidermal growth factor (0.01 μg ml^{-1}), cholera toxin (0.01 μg ml^{-1}), hydrocortisone (0.4 μg ml^{-1}), penicillin (10^5 units liter^{-1}), and streptomycin (100 mg liter^{-1}). W12 cells were maintained in DMEM, 10% FBS, cholera toxin (0.01 nM), hydrocortisone (0.4 μg ml^{-1}), epidermal growth factor (0.01 μg ml^{-1}), penicillin (10^5 units liter^{-1}), and streptomycin (100 mg liter^{-1}). Cells were maintained in a humidified atmosphere at 37°C and 5% CO_2. Twenty-four hours prior to transfection, cells were seeded at 3×10^5 cells per well onto coverslips in six-well plates and incubated overnight. The cells were then transiently transfected using Tfx-20 (Promega) for Saos-2, 879, CaSki, and 873F cells, Tfx-50 (Promega) for NIH 3T3 and SiHa cells, and Fugene 6 (Roche Molecular Biochemicals) for the remaining cell lines.

Apoptosis assays. Cells were seeded onto coverslips in six-well plates and incubated overnight to obtain subconfluent cultures. The cells were then transiently cotransfected with E2 expression plasmids and red fluorescent protein (RFP) or green fluorescent protein (GFP) expression plasmids using Fugene 6 (Roche Molecular Biochemicals). After 30 h, the coverslips were washed in phosphate-buffered saline and assayed for apoptosis using the Dead-End Fluorometric TUNEL (terminal uridyl-nucleotide end labeling) system (Promega). Nuclear morphology was assessed by staining with bisbenzimide (Hoechst no. 33258; Sigma) at 22°C for 30 min. Transfected cells were identified on the basis of RFP fluorescence in the case of TUNEL assays and GFP fluorescence in other experiments. Microscopy was carried out using a Leica DM IRBE inverted epifluorescent microscope with tetramethyl rhodamine isothiocyanate (RFP), fluorescein isothiocyanate (GFP), and 4′,6′-diamidino-2-phenylindole (DAPI) (bisbenzimide) filter sets and a 20× air objective (Leica).

Transcription assays. Cells were transiently transfected using Fugene 6 (Roche Molecular Biochemicals). After 24 h, the media were removed and the cells were washed twice with phosphate-buffered saline. Luciferase activity was then determined using a Dual-Luciferase assay system (Promega) according to the manufacturer's instructions. The *Renilla* luciferase expressing the plasmid pRL-CMV (100 ng) was included in each transfection as an internal control for transfection efficiency.

Western blotting. HeLa and COS-7 cells (1×10^6) were transiently transfected as described above. Twenty-four hours posttransfection, the cells were lysed using boiling 2× concentrated sodium dodecyl sulfate (SDS) loading buffer containing protease inhibitors (Complete protease inhibitor tablet; Roche Molecular Biochemicals). The proteins were separated on 10% SDS–polyacrylamide gel electrophoresis (PAGE) gels before being transferred to a polyvinylidene difluoride (PVDF) membrane. GFP-tagged proteins were visualized using a GFP-specific monoclonal antibody (Covance), a horseradish peroxidase-conjugated anti-mouse secondary antibody (Santa Cruz), and BM chemiluminescence blotting substrate (Roche Molecular Biochemicals).

DNAs used in this study. The eukaryotic expression vector pWEB-E2 expresses the wild-type HR-HPV16 E2 protein under the control of the cytomegalovirus (CMV) promoter (45). Plasmids pWEB-11 E2 and pWEB-6 E2 were produced by cloning the relevant cDNA sequences in place of the HR-HPV16 cDNA in pWEB-E2. pEGFP-16 E2 expresses the HR-HPV16 E2 protein with an N-terminal enhanced GFP (eGFP) tag and was kindly provided by Agnieszka Olejnik (Poznań University of Agriculture, Poland). pEGFP-11 E2 and pEGFP-6 E2 were produced by cloning the relevant cDNA sequences in place of the HR-HPV16 cDNA in pEGFP-16 E2. Plasmid pCMX-GFP3 expresses green fluorescent protein and was supplied by Jeremy Tavaré (University of Bristol, United Kingdom). pHcRED-C1 expresses red fluorescent protein (Clontech). The E2-reponsive thymidine kinase 6E2-luciferase reporter plasmid was supplied by Iain Morgan (University of Glasgow, United Kingdom). The p53-responsive mdm2-luciferase reporter plasmid pGL2NA(mdm2) and the p53 expression vectors pCB6-p53 and pCB6-p53.175P were supplied by Karen Vousden (Beatson Institute, United Kingdom). The p53 expression vectors pC53.SN3 (wild-type p53), pC53.143A, pC53.175H, pC53.248W, and pC53.273H were supplied by Bert Vogelstein (Johns Hopkins Oncology Center).

The bacterial expression vector pKK-16E2Ct expresses the C-terminal 86 amino acids of the HR-HPV16 E2 protein (45). This fragment of E2 corresponds to the minimal DNA-binding domain. The equivalent 90-amino-acid domains from the LR-HPV6 and LR-HPV11 proteins were expressed in bacteria by cloning the relevant coding sequences into pKK223-3 (Pharmacia Biotech) to create pKK-6E2Ct and pKK-11E2Ct, respectively. To create the HR-HPV16 $E2_{p53m}$Ct mutant Trp341, Asp344 and Asp338 in HR-HPV16 E2 were replaced by alanines. To do this, the PstI-HindIII fragment encoding the C-terminal portion of the E2Ct protein in pKK-16E2Ct was replaced with three mutant double-stranded synthetic oligonucleotides. pKK-16$E2_{DBDm}$Ct expresses a DNA-binding-defective HR-HPV16 E2Ct protein in which amino acids Asn296, Lys299, and Arg304 have been mutated to alanines (45). The p53m and DBDm mutations were combined by cloning the PstI-HindIII fragment from the pKK-16$E2_{p53m}$Ct construct between the PstI and HindIII sites in pKK-16$E2_{DBDm}$Ct to create pKK-16$E2_{p53mDBDm}$Ct. DNA sequences encoding the $E2_{p53m}$ mutations were excised from pKK-16$E2_{p53m}$Ct using PstI and HindIII and used to replace the equivalent HR-HPV16 E2 sequence in pWEB-E2 and pWEB-$E2_{DBDm}$ to create the eukaryotic expression vectors pWEB-$E2_{p53m}$ and pWEB-$E2_{p53mDBDm}$, respectively. All constructs were verified by DNA sequencing.

Molecular modeling. The crystal structure of the p53-53BP2 complex (PDB 1YCS) shows a tryptophan residue from 53BP2 (Trp498) in the center of the dimer interface (see Fig. 5A). Helix 2 in the DNA-binding domain of the HR-HPV16 E2 protein (PDB 1BY9) also contains a tryptophan residue (Trp341). A model of the p53-E2 complex (see Fig. 5B) was generated by superimposing Trp341 of E2 upon Trp498 of 53BP2 in the p53-53BP2 complex using InsightII v97 (MSI, San Diego, Calif.).

Proteins. *Escherichia coli* XL1-Blue cells containing E2 expression plasmids were grown to an optical density at 600 nm of 0.5, and protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C overnight. After harvesting by centrifugation, the cells were lysed by sonication at 4°C and the proteins purified over an SP-Sepharose FF cation-exchange medium column followed by a MonoS HR 16/10 cation-exchange fast-performance liquid chromatography column as described previously (45). A glutathione *S*-transferase (GST)–p53 fusion protein containing the full-length human p53 protein was expressed as described above and purified over a glutathione-Sepharose 4B column followed by a HiLoad 16/60 Superdex gel filtration column.

Filter binding assays. Equal amounts (250 pM) of the purified HR-HPV16 E2Ct, LR-HPV6 E2Ct, and LR-HPV11 E2Ct proteins were immobilized on a PVDF membrane by slot blotting. In vitro transcription and translation of p53 was carried out using a TNT kit (Promega) according to the manufacturer's protocol. The membrane was incubated with ^{35}S-labeled p53 for 90 min at 22°C in Tris-buffered saline, 0.02% (vol/vol) Tween 20, 10% (wt/vol) dried skim

milk powder, and after washing, bound protein was visualized using a PhosphorImager. A duplicate membrane was incubated with a ^{32}P-labeled 20-bp oligonucleotide carrying E2 site 4 from the HR-HPV16 genome (see below) and bound DNA was visualized as described above.

Surface plasmon resonance. GST antibodies were immobilized on a CM5 chip using an amine coupling kit (Biacore AB) and a Biacore 2000 apparatus. GST-p53 and E2 proteins were flowed across the immobilized construct at 20 μl/min and 25°C. Surfaces were regenerated by injecting a pulse of 10 mM glycine-HCl, pH 2.0, at 20 μl/min. Apparent association rate constants (k_a, M^{-1} s^{-1}) and dissociation constants (K_D, s^{-1}) were calculated from the respective association and dissociation curves. The overall dissociation constant is assumed to be the ratio of K_D/k_a and is used here simply to compare the wild-type and mutant proteins.

Gel retardation assays. Increasing amounts of each E2 protein were incubated with a γ-P^{32}-labeled double-stranded oligonucleotide corresponding to HR-HPV16 E2 binding site 1 (5′-TTGAACCGAAACCGGTTAGT-3′) in 20 mM HEPES, pH 7.9, 25 mM KCl, 1 mM dithiothreitol, 0.1% Nonidet P-40, 10% glycerol, and 0.5 μg/μl bovine serum albumin. After 20 min at 20°C, free and bound labeled DNA were resolved on 5% nondenaturing polyacrylamide gels run in 1× Tris-buffered EDTA and quantified using a PhosphorImager and Molecular Dynamics ImageQuant software. The apparent equilibrium constant, $K_{eq(apparent)}$, was obtained using the following equation: $[_{bound}DNA] = [_{maximum\ bound}DNA][protein]/[protein] + K_{eq(apparent)}$.

RESULTS

LR-HPV E2 proteins fail to induce apoptosis. HPV16 E2-induced apoptosis has been confirmed using a variety of techniques, including the direct observation of cell morphology, TUNEL assays, annexin V staining, caspase assays, and flow cytometry (10, 11, 40). To determine whether the E2 proteins from LR-HPV types might also induce apoptosis, we transiently transfected plasmids expressing the LR-HPV6, LR-HPV11, and HR-HPV16 E2 proteins into HeLa cells growing on coverslips. HeLa cells are an HPV18-transformed cell line that contains integrated viral genomes and does not express the HPV18 E2 protein. We then used a TUNEL assay to count the number of apoptotic cells. Increasing amounts of E2 expression vectors were transiently cotransfected into these cells using lipofection along with a plasmid that expresses the red fluorescent protein. Mixing of the DNAs prior to encapsulation in liposomes makes it very likely that the transfected cells receive both plasmids. After 30 h, the cells were fixed and their DNA was stained with bisbenzimide (Hoechst stain). Transfected cells were identified on the basis of their red fluorescence and examined for apoptosis using the TUNEL assay (Fig. 1A). At least 100 transfected cells were counted on each coverslip, and the experiment was repeated three times. The percentage of apoptotic cells in the transfected population rises as increasing amounts of the HR-HPV16 E2 expression vector are added (Fig. 1B). In contrast, increasing amounts of the LR-HPV6 or LR-HPV11 E2 expression vectors have no effect on the levels of apoptosis seen in the transfected cells (Fig. 1B). The same results were obtained using GFP to detect transfected cells and the direct assessment of cell morphology to count apoptotic cells (data not shown).

Although our data suggest that the LR-HPV6 and LR-HPV11 E2 proteins do not induce apoptosis, another possibility is that these E2 proteins are not being expressed in the transfected cells. However, western analysis using tagged proteins confirms that all three E2 proteins are expressed at equivalent levels (Fig. 1C). Cos-7 cells were used in this experiment because in HeLa cells the HR-HPV E2 protein is proteolytically cleaved during E2-induced apoptosis, making the comparison of E2 expression levels in these cells problematic (10). Since Cos-7 cells do not undergo E2-induced apoptosis, we were able to compare the expression levels of the tagged proteins in these cells. We presume that the small differences in protein mobility seen in the Western blot result from post-translational modifications of the proteins and, in particular, from differences in phosphorylation. To confirm that the proteins are expressed in HeLa cells at roughly equivalent levels, we assayed their ability to activate transcription from an E2-dependent promoter. The tagged E2 proteins activate transcription in HeLa cells, confirming that they are biologically active (Fig. 1D). Previous studies have shown that, at equivalent expression levels, the LR-HPV E2 proteins activate transcription less well than the HR-HPV E2 proteins (21), and this is also seen with the tagged E2 proteins. The tagged proteins activate transcription to the same degree as the untagged proteins, and in the case of the HR-HPV16 E2 protein, the tagged

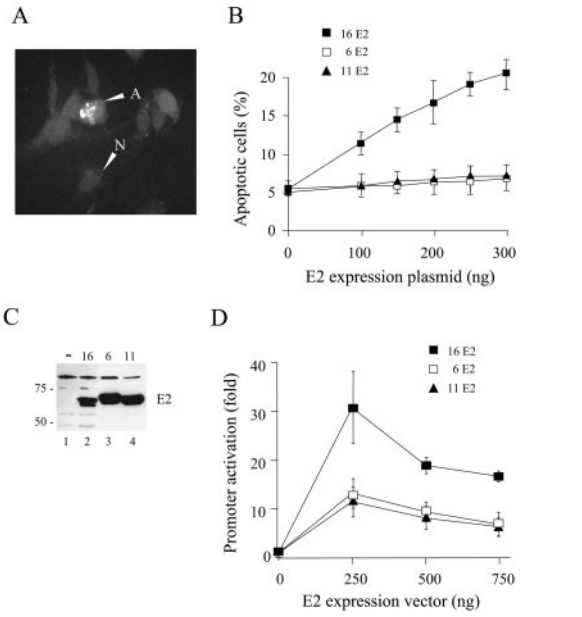


FIG. 1. LR-HPV E2 proteins fail to induce apoptosis. (A) A plasmid expressing HR-HPV16 E2 was transiently cotransfected into HeLa cells along with a plasmid that expresses RFP (pHcRED-C1). After 30 h, apoptotic cells in the transfected population were identified using a TUNEL assay with fluorescein-12-dUTP. A, apoptotic nucleus; N, normal nucleus. (B) pHcRED-C1 and plasmids expressing HR-HPV16, LR-HPV6, or LR-HPV11 E2 were transiently cotransfected into HeLa cells, and transfected apoptotic cells were counted as described above. The transfection was performed in duplicate and repeated three times, and the data are the means and standard deviations. (C) Cos-7 cells were transiently transfected with plasmids expressing HR-HPV16 E2 (lane 2), LR-HPV6 E2 (lane 3), or LR-HPV11 E2 (lane 4) fused to eGFP or left untransfected (lane 1). After 24 h, whole-cell lysates were separated by SDS-PAGE and transferred to a PVDF membrane. The tagged proteins were detected using anti-eGFP monoclonal antibodies (Covance). The sizes of the markers used are indicated. (D) HeLa cells were transiently cotransfected with an E2 responsive luciferase reporter plasmid and the E2 expression vectors described above. Luciferase activity was normalized for transfection efficiency using a cotransfected plasmid expressing *Renilla* luciferase. The data are presented as promoter activity relative to the reporter alone and are the means and standard deviations of results from three independent experiments.

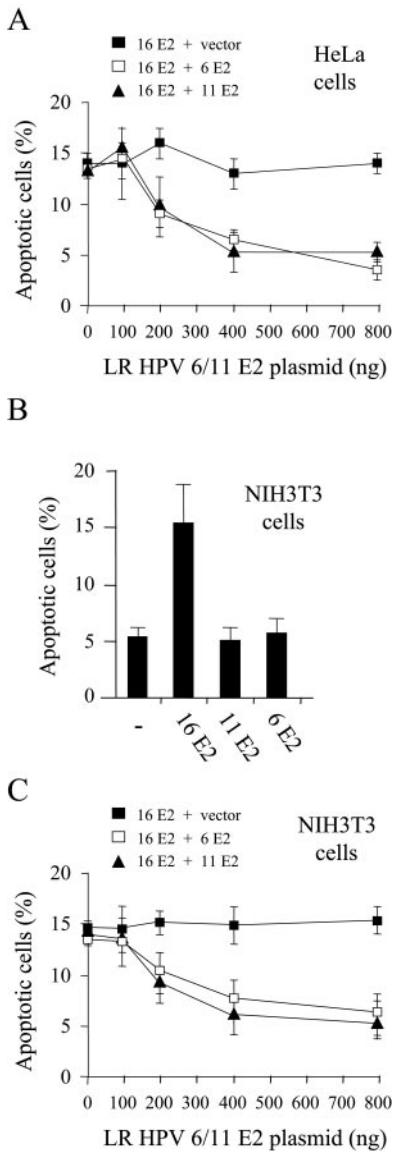


FIG. 2. LR-HPV E2 proteins inhibit HR-HPV16 E2-induced apoptosis. (A) pCMX-GFP3 and 200 ng of a plasmid expressing HR-HPV16 E2 were transiently cotransfected into HeLa cells along with increasing amounts of plasmids expressing either LR-HPV6 E2 or LR-HPV11 E2 or increasing amounts of the empty vector. The percentage of apoptotic cells in the transfected population was determined by counting. (B) pCMX-GFP3 and plasmids expressing HR-HPV16, LR-HPV6, or LR-HPV11 E2 were transiently cotransfected into NIH 3T3 cells, and transfected apoptotic cells were counted as described above. The transfection was performed in duplicate and repeated three times, and the data are means and standard deviations. (C) The experiment described for panel A was repeated in NIH 3T3 cells.

and untagged proteins both induce apoptosis in HeLa cells (data not shown).

LR-HPV E2 proteins block HPV16 E2-induced apoptosis. We wondered whether overexpression of the LR-HPV E2 proteins might augment or inhibit HR-HPV E2-induced apoptosis. When a fixed amount of the HR-HPV16 E2 expression vector is cotransfected into HeLa cells along with increasing amounts of either the LR-HPV6 or LR-HPV11 E2 expression vectors, there is a decrease in the levels of HR-HPV16 E2-induced cell death (Fig. 2A). In contrast, increasing amounts of an empty expression vector has little or no effect on the level of HR-HPV16 E2-induced cell death (top line in Fig. 2A). Thus, overexpression of the LR-HPV6 E2 or LR-HPV11 E2 protein can block HR-HPV16 E2-induced apoptosis in these HPV-transformed cells. One possible explanation for this observation could be that the LR-HPV E2 proteins compete with the HR-HPV E2 protein for binding sites within the integrated HPV genome and thereby alter the expression of E6 and/or E7. To investigate this possibility, we next looked at the ability of the LR-HPV E2 proteins to block HR-HPV16 E2-induced apoptosis in HPV-negative cells.

We have shown previously that the HR-HPV16 E2 protein can induce apoptosis in NIH 3T3 cells, an HPV-negative mouse fibroblast cell line (45). Although the HR-HPV16 E2 protein induces apoptosis in these cells, the LR-HPV6 and LR-HPV11 E2 proteins do not increase the level of apoptosis above that seen in cells transfected with a GFP expression vector alone (Fig. 2B). As in the previous experiment, the addition of either LR-HPV6 or LR-HPV11 E2 expression vector blocks HR-HPV16 E2-induced cell death (Fig. 2C). Taken together with the data described above, these results confirm that the LR-HPV6 and LR-HPV11 E2 proteins are expressed, that they fail to induce apoptosis, and that they can block HR-HPV16 E2-induced cell death in both HPV-transformed and non-HPV-transformed cell lines.

HR-HPV16 E2 induces p53-activated transcription. Wild-type p53 activates transcription from the mdm2 promoter in response to proapoptotic signals. To determine whether the different E2 proteins induce p53-activated transcription, we cotransfected HeLa cells with E2 expression vectors and an mdm2-luciferase reporter plasmid. Cotransfection of the mdm2-luciferase reporter with increasing amounts of the HR-HPV16 E2 expression vector brings about a threefold increase in luciferase activity (Fig. 3A). In contrast, cotransfection of the mdm2-luciferase reporter with the LR-HPV E2 expression vectors has no effect on luciferase activity. Since HeLa cells are an HPV18-transformed cell line, it is possible that differences in the DNA-binding or transcriptional activities of the HR and LR proteins could account for the difference in the ability of these proteins to induce p53-activated transcription in these cells. To investigate this possibility, we repeated the above experiment using Saos-2 cells. Saos-2 cells are a human osteosarcoma cell line that is p53 null. The HR-HPV16 E2 protein alone fails to induce mdm2 promoter activity in these cells (Fig. 3B). As might be expected, transfection of these cells with the p53 expression vector pCB6-p53 results in a significant increase in mdm2 promoter activity. Interestingly, cotransfection of these cells with the p53 expression vector and increasing amounts of the HR-HPV16 E2 expression vector results in a further increase in mdm2 promoter activity (Fig. 3B). In contrast, neither of the LR-HPV E2 proteins bring about this increase in p53-activated transcription (Fig. 3B). Thus, these experiments show that the HR-HPV16 E2 protein can induce p53 activity in both HPV-transformed and non-HPV-transformed cell lines.

The data presented above suggest that p53 plays a role in HR-HPV E2-induced apoptosis. This is consistent with our previous work which showed that overexpression of the p53

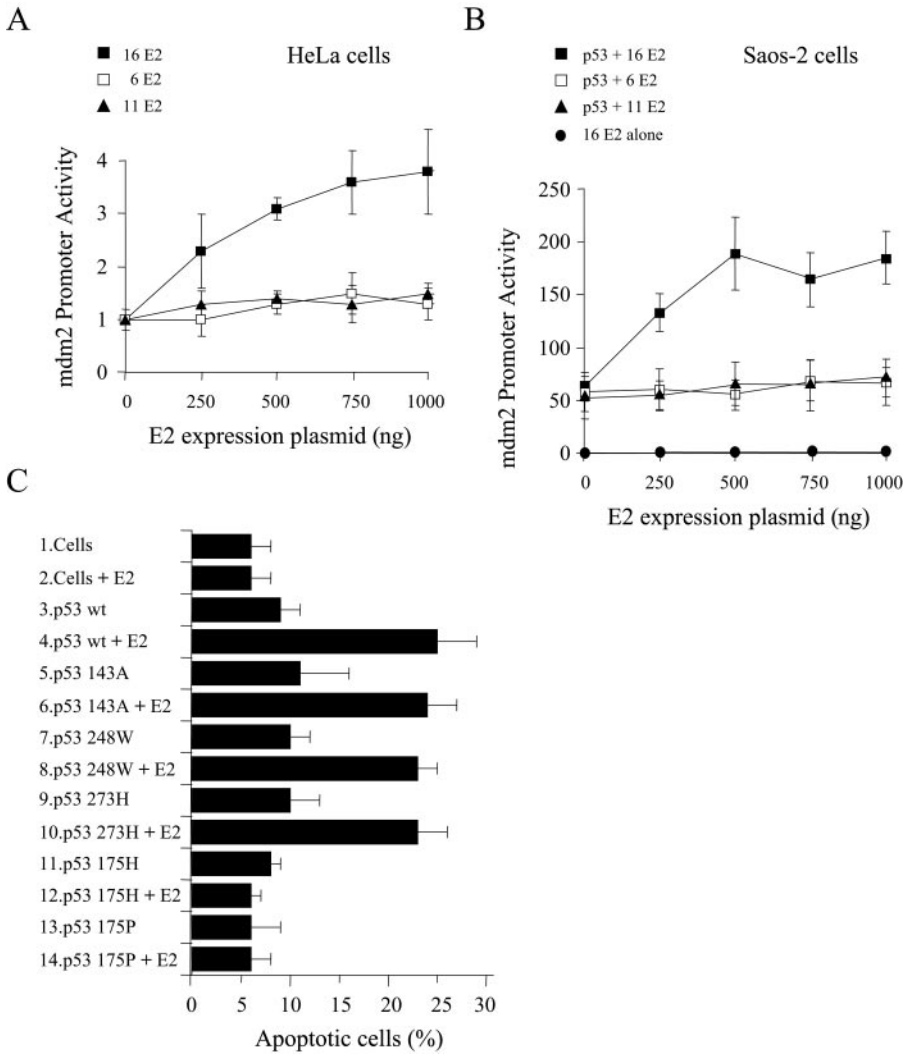


FIG. 3. The role of p53 in HR-HPV16 E2-induced apoptosis. (A) HeLa cells were transiently cotransfected with an mdm2-luciferase reporter plasmid and E2 expression vectors. Luciferase activity was normalized for transfection efficiency using a cotransfected plasmid expressing *Renilla* luciferase. The data are presented as promoter activity relative to the mdm2-luciferase reporter alone and are means and standard deviations of results from four independent experiments. (B) Saos-2 cells were transiently cotransfected with the mdm2-luciferase reporter plasmid, the p53 expression vector pCB6-p53 and E2 expression vectors. Luciferase activity was normalized for transfection efficiency as described above, and results are means and standard deviations of results from four independent experiments. (C) Saos-2 cells were transiently transfected with plasmids expressing GFP, HR-HPV16 E2, and a panel of p53 proteins. Apoptotic cells were identified as described previously. The transfection was performed in duplicate and repeated three times. The data are means and standard deviations.

protein in HeLa cells increases the levels of HPV16 E2-induced apoptosis, whereas overexpression of a *trans*-dominant-negative p53 mutant blocks E2-induced apoptosis (45). However, definitive confirmation of the role of p53 in HPV16 E2-induced apoptosis is lacking. To throw light on this question, we made further use of p53-null Saos-2 cells The HR-HPV16 E2 expression vector was transiently cotransfected into Saos-2 cells growing on coverslips along with a plasmid that expresses wild-type p53. As before, a plasmid that expresses RFP was included in each experiment to allow the identification of transfected cells. Transfection of Saos-2 cells with pCB6-p53 induces significant levels of apoptosis (not shown). However, low amounts of pCB6-p53 (200 ng) bring about only a modest increase in the level of apoptosis (compare columns 1 and 3 in Fig. 3C). Transfection with a plasmid expressing the HR-HPV16 E2 protein has no effect on the level of apoptotic Saos-2 cells (Fig. 3C, column 2). In contrast, cotransfection with plasmids expressing HPV16 E2 and p53 increases the level of apoptotic cells to over 25% of the transfected population (Fig. 3C, column 4). These results confirm that the HR-HPV16 E2 protein is capable of inducing both p53-dependent apoptosis and p53-activated transcription. In contrast, the LR-HPV E2 proteins induce neither p53-dependent apoptosis nor p53-activated transcription.

Mutant p53 proteins can mediate HR-HPV16 E2-induced apoptosis. To determine whether the ability of p53 to regulate transcription is required for E2-induced apoptosis, we next looked at the ability of the HR-HPV16 E2 protein to induce p53-dependent apoptosis in conjunction with a panel of well-characterized p53 mutants. Saos-2 cells were transiently with

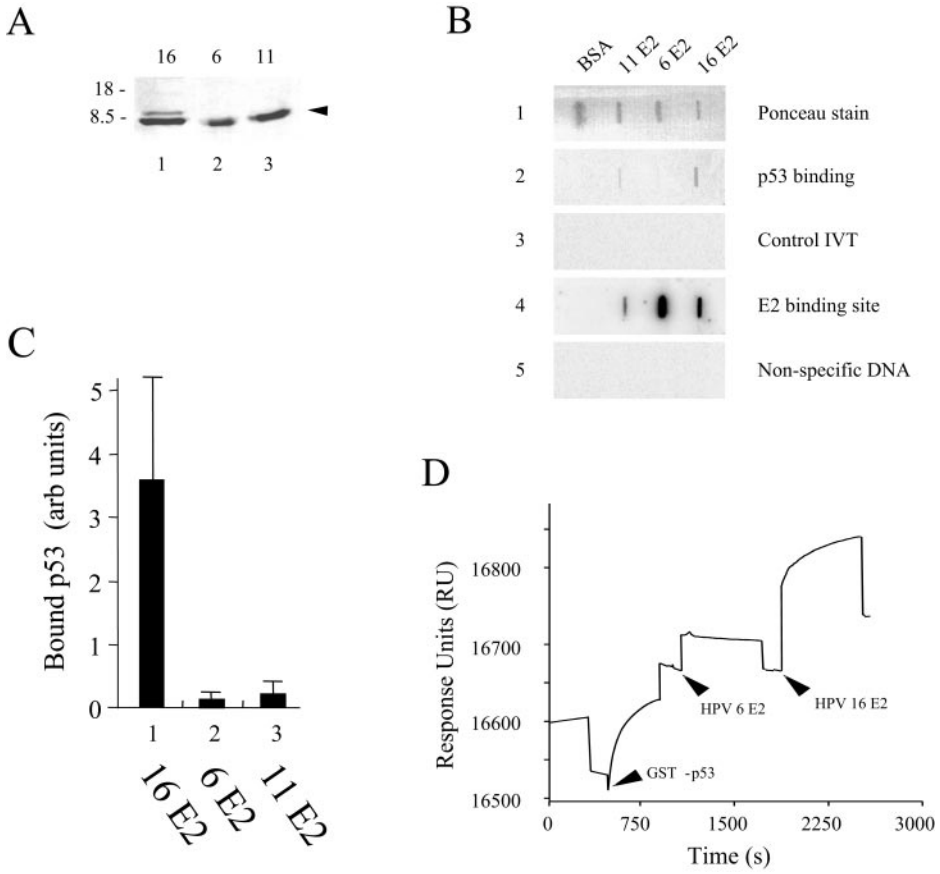


FIG. 4. LR-HPV6 and LR-HPV11 E2 bind poorly to p53. (A) Samples of the purified HR-HPV16 E2Ct, LR-HPV6 E2Ct, and LR-HPV11 E2Ct proteins were analyzed by SDS-PAGE (indicated by the arrowhead). The sizes of the markers used are indicated to the left of the gel. (B) Equal amounts (250 pM) of the purified HR-HPV16 E2Ct, LR-HPV6 E2Ct, and LR-HPV11 E2Ct proteins were immobilized on a PVDF membrane by slot blotting. The membrane was then incubated with ^{35}S-labeled p53, and after extensive washing, the bound protein was visualized using a PhosphorImager (panel 2). After washing to remove the bound p53, the membrane was incubated with an unrelated ^{35}S-labeled protein (panel 3). A duplicate membrane was incubated with a ^{32}P-labeled 20-bp oligonucleotide carrying an E2 binding site (panel 4) or a ^{32}P-labeled unrelated oligonucleotide (panel 5), and bound DNA was visualized as described above. (C) The graph shows the results of three filter-binding experiments performed in duplicate. The data shown are averages and standard deviations. (D) SPR analysis of LR-HPV6 E2Ct and HR-HPV16 E2Ct binding to immobilized GST-p53. A GST-p53 fusion protein was immobilized through amine coupling on a CM5 sensor chip. LR-HPV6 E2Ct was then passed over the immobilized GST-p53. After reequilibration, HR-HPV16 E2Ct was passed over the immobilized GST-p53. As can be seen from the data, HR-HPV16 E2Ct brings about a significant increase in the SPR signal, whereas LR-HPV6 E2Ct has little or no effect.

plasmids expressing HR-HPV16 E2 and either wild-type or mutated p53 (Fig. 3B). p53 143A, p53 248W, and p53 273H are mutants of p53 that are unable to bind DNA or activate transcription (27). However, each of these p53 mutants is capable of inducing apoptosis when cotransfected into Saos-2 cells along with the HR-HPV16 E2 protein (Fig. 3B, columns 5 to 10). p53 175P and p53 175H are transcriptionally active and transcriptionally inactive mutants of p53, respectively (31). Neither of these mutants induces apoptosis when cotransfected with the HR-HPV16 E2 expression vector (Fig. 3B, columns 11 to 14). These results suggest that although the HR-HPV16 E2 protein induces p53-activated transcription, the ability of p53 to activate or repress transcription is not required for this protein to induce p53-dependent apoptosis in Saos-2 cells.

LR-HPV E2 proteins fail to bind p53. The C-terminal DNA binding domain of the HR-HPV16 E2 protein has been shown to interact with p53 in vitro using GST pull-down experiments and in cells using coimmunoprecipitation assays (32). To determine whether the E2 proteins from LR-HPV6 and LR-HPV11 can also interact with p53, we expressed the C-terminal domains (E2Ct) of these proteins in bacteria and tested their ability to bind ^{35}S-labeled p53. Equal amounts of the HR-HPV16 E2Ct, LR-HPV6 E2Ct, and LR-HPV11 E2Ct proteins (Fig. 4A) were immobilized on a PVDF membrane by slot blotting (Fig. 4B). This approach makes use of untagged proteins and thereby avoids any nonspecific binding to tags or beads that can make pull-down experiments and coimmunoprecipitation assays difficult to interpret. The membrane was incubated with in vitro-transcribed and -translated p53, and bound protein was visualized using a PhosphorImager. Labeled p53 is retained by the HR-HPV16 E2 protein but binds poorly to the LR-HPV6 E2 and LR-HPV11 E2 proteins (Fig. 4B, panel 2). To confirm that the retained material is p53 and not some other component of the reaction mix, we washed the membrane to remove the bound proteins and then repeated the experiment using an in vitro transcription/translation lysate

programmed to express an unrelated protein. In this case, no labeled proteins are retained on the membrane (Fig. 4B, panel 3). To confirm that the immobilized E2 proteins are correctly folded, we incubated a duplicate membrane with a ^{32}P-labeled oligonucleotide carrying an E2 binding site. The E2 proteins retained labeled DNA (Fig. 4B, panel 4), showing that they are functional. However, the amount of retained labeled DNA varies in each case due to differences in the DNA binding properties of these proteins (8). To ensure that the observed DNA binding is specific, we washed the membrane to remove bound E2 sites and then repeated the experiment using an unrelated labeled oligonucleotide. The unrelated labeled oligonucleotide is not retained on the membrane (Fig. 4B, panel 5). Since equal amounts of the E2 proteins were immobilized on the membrane, these data suggest that p53 binds more tightly to the HR-HPV16 E2 protein than it does to the E2 proteins from LR-HPV6 or LR-HPV11.

The data from several filter binding experiments were quantified using a PhosphorImager (Fig. 4C). These data suggest that at least 10-fold-more p53 binds to the HR-HPV16 E2 protein than binds to the LR-HPV E2 proteins. Further confirmation of this result was obtained using surface plasmon resonance (SPR). In this technique, protein-protein interactions are detected by monitoring changes in the refractive index at a surface to which one of the partners is attached. Protein association and dissociation are recorded as an increase in response as proteins in solution are flowed across the immobilized ligand and a subsequent decrease in response as bound proteins are released from the immobilized ligand. A GST-p53 fusion protein containing the full-length human p53 protein was immobilized on a sensor chip using amine coupling. GST-p53 was then flowed across the immobilized GST antibodies. LR-HPV6 E2Ct and HR-HPV16 E2Ct proteins were subsequently flowed across the immobilized GST-p53 under the same conditions. As can be seen from the data shown in Fig. 4D, the HR-HPV16 E2Ct protein binds to the immobilized p53, causing an increase in response. In contrast, the LR-HPV6 E2Ct protein fails to bind to the immobilized p53. The same result was obtained using the LR-HPV11 E2Ct protein (data not shown).

Disruption of the E2-p53 interaction. To determine whether the interaction between HR-HPV16 E2 and p53 is required for E2-induced apoptosis, we used site-directed mutagenesis to mutate the HR-HPV16 E2 protein at positions predicted to be critical for binding to p53. The C-terminal DNA binding/dimerization domain of the HPV16 E2 protein binds to p53 (32), and the structure of this domain of E2 has been determined by X-ray crystallography (20). To identify amino acids in E2 that might contact p53, we made a molecular model of the E2-p53 interaction (Fig. 5A and 5B) using the crystal structure of p53 bound to the p53-binding protein 53BP2 as a guide (19). The modeling identified several amino acids in the E2 protein that can be superimposed on amino acids present in 53BP2 and known to be important in the p53-53BP2 interaction. Trp498 in the p53-53BP2 complex is flanked by three acidic residues, Glu495, Glu497, and Asp492. These three residues are mimicked in the modeled p53-E2 interface by Glu340, Asp344, and Asp338 from E2 (Fig. 5C). Trp341, Asp344, and Asp338 in E2 were therefore simultaneously mutated to alanine in the hope of disrupting the binding of E2 to p53. Glu340 is conserved between E2 proteins from HR- and LR-HPV types and was therefore not mutated. Trp341, Asp344, and Asp338 are not conserved between HR-HPV16 E2 and the LR-HPV11 and LR-HPV6 E2 proteins, and this is consistent with the inability of the latter proteins to bind p53.

The wild-type and mutated HPV16 E2Ct proteins were expressed in bacteria and purified to homogeneity. The binding of these proteins to p53 was then examined using SPR (Fig. 5D and 5E). The mutations impair the ability of E2Ct to bind to a GST-p53 fusion protein. Calculated apparent rate constants are shown in the inset in Fig. 5D. The slower k_a for the mutant suggests that the ability of this protein to recognize p53 is impaired. Neither protein binds to GST alone (data not shown). To determine whether the mutations alter the stability or folding of the E2Ct protein, we compared the DNA binding activity of the wild-type and mutated proteins (Fig. 5F). There is no significant difference in the affinity of these proteins for an E2 binding site [the $K_{\mathrm{eq(apparent)}}$ for the wild-type and mutated proteins are 15 nM and 11 nM, respectively]. Thus, the mutations have little or no effect on protein folding or stability. Interestingly, the electrophoretic mobility of the protein-DNA complexes containing the wild-type and mutated E2 proteins are different, possibly due to differences in the charge of the proteins.

HR-HPV16 E2 induces apoptosis by at least two pathways. We have described previously a mutated HR-HPV16 E2 protein that is unable to bind DNA (45). We combined the mutations described above that inhibit binding to p53 with mutations that block DNA binding to create the series of proteins shown in Fig. 6A. The wild-type E2Ct protein binds to both p53 and DNA, the $E2_{\mathrm{DBDm}}$Ct protein is defective in DNA binding, the $E2_{\mathrm{p53m}}$Ct protein is defective in p53 binding, and the $E2_{\mathrm{p53mDBDm}}$Ct double mutant is defective in both p53 and DNA binding. To test the ability of E2 proteins carrying these mutations to induce apoptosis, we introduced the same mutations into the full-length HR-HPV16 E2 protein and transfected the resulting constructs into 5 HPV-transformed cell lines and 5 non-HPV-transformed cell lines. The results of these experiments are shown in Fig. 6B. As expected, the wild-type E2 protein induces apoptosis in all of the cell lines tested (Fig. 6B, column 4). Similarly, the $E2_{\mathrm{DBDm}}$ protein induces apoptosis in all of the cell lines (Fig. 6B, column 5). These data confirm our previous observation that the DNA binding activity of E2 is not required for the induction of apoptosis (45). In marked contrast, the $E2_{\mathrm{p53m}}$ protein induces apoptosis in all of the HPV-transformed cell lines but fails to induce apoptosis in the non-HPV-transformed cell lines (Fig. 6B, column 6). Since the $E2_{\mathrm{p53m}}$ protein induces apoptosis in the HPV-transformed cell lines, the protein must be expressed and correctly folded. These data suggest that the E2-p53 interaction is required for E2-induced apoptosis in non-HPV-transformed cells but is not required for E2-induced apoptosis in HPV-transformed cells. The $E2_{\mathrm{p53mDBDm}}$ protein fails to induce apoptosis in any of the cell lines tested (Fig. 6B, column 7). The $E2_{\mathrm{p53mDBDm}}$ protein is able to block apoptosis induced by the wild-type E2 protein (data not shown), indicating that the mutant protein is expressed and confirming that the loss of both DNA binding activity and p53 binding activity completely abrogates the proapoptotic effects of this protein.

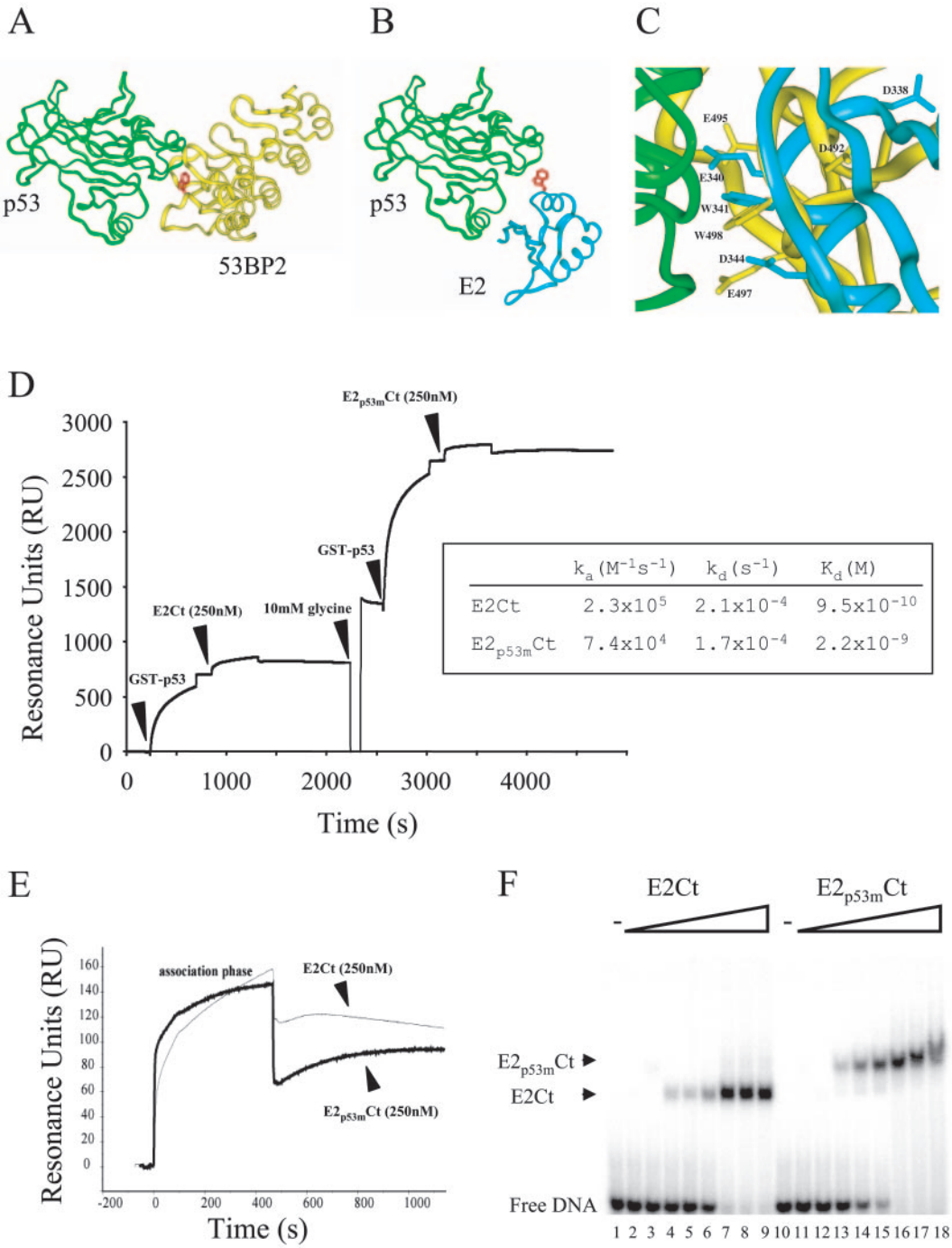
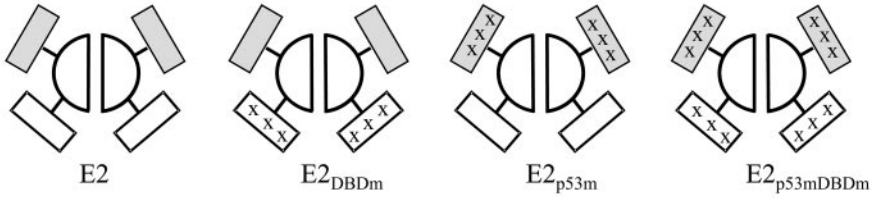


FIG. 5. Disruption of the E2-p53 interaction using site-directed mutagenesis. (A) Crystal structure of p53 (green) bound to 53BP2 (yellow). W498 in 53BP2 is highlighted in red. (B) Molecular model of p53 (green) bound to a monomer of HPV16 E2 (blue). W341 in E2 is highlighted in red. (C) The putative interface between p53 (green) and E2 (blue) is overlaid on the p53-BP2 interface. Amino acids E495, E497, and D492 in 53BP2 are mimicked by E340, D344, and D338 from E2. (D) SPR analysis of HR-HPV16 E2Ct and $E2_{p53m}Ct$ binding to immobilized GST-p53. The apparent association rate constants (k_a, $M^{-1}s^{-1}$) and dissociation constants (K_D, s^{-1}) were calculated from the respective association and dissociation curves (E). The values obtained are relative, since the dissociation of the immobilized GST-p53, although an order of magnitude slower than that of retained E2Ct or $E2_{p53m}Ct$, was not taken into consideration. (F) Labeled oligonucleotides carrying an E2 binding site were incubated with 0, 2, 5, 10, 25, 50, 125, 250, and 500 nM purified E2Ct (lanes 1 to 9) or $E2_{p53m}Ct$ (lanes 10 to 18). Free and bound DNA were separated on a 5% polyacrylamide gel and then visualized and quantified using a PhosphorImager.

A



B

Cell Line	HPV status	Apoptosis (%) pWEB	E2	$E2_{DBDm}$	$E2_{p53m}$	$E2_{p53mDBDm}$
HeLa	HPV 18	3±2	19±2	20±2	27±4	4±2
SiHa	HPV 16	5±3	32±4	33±5	37±5	5±3
879	HPV 16	9±4	35±3	40±4	38±4	8±2
CaSki	HPV 16	11±3	31±6	39±3	36±3	11±2
W12	HPV 16	5±1	16±2	25±2	22±2	6±1
808F	-	7±2	19±2	25±2	10±1	9±1
873F	-	3±1	13±2	20±2	3±1	5±2
NIH3T3	-	2±1	16±2	18±2	5±1	3±3
MCF-7	-	6±1	23±3	25±3	10±1	6±1
Saos-2*	-	10±1	28±6	24±5	8±1	8±1

FIG. 6. E2p53m induces apoptosis in HPV-positive cells. (A) A schematic summary of the E2 mutants used in this work. Each E2 protein is a dimer. The DNA recognition helices are represented by open rectangles, while the putative p53 interfaces are represented by filled rectangles. $E2_{DBDm}$ contains mutations that block binding to DNA (N296A, K299A, and R304A). $E2_{p53m}$ contains mutations that decrease binding to p53 (W341A, D344A, and D338A). $E2_{p53mDBDm}$ contains both sets of mutations. (B) Plasmids expressing the E2 proteins described above (600 ng) were transiently cotransfected into the cell lines listed along with GFP expression vector. At 30 h posttransfection, the percentage of apoptotic cells was determined as before. Soas-2* cells are p53-null and were additionally cotransfected with 200 ng of the p53-expressing plasmid pCB6-p53.

DISCUSSION

We have shown that the E2 proteins from LR-HPV6 and LR-HPV11 fail to induce apoptosis. Somewhat surprisingly, the E2 proteins from these LR-HPV types are able to block HR-HPV16 E2-induced apoptosis. Since all of the E2 proteins bind to essentially the same DNA sequences (30, 43), it is possible that the E2 proteins from the LR viral types might compete with the HR-HPV16 E2 protein for E2 binding sites within the HPV genome. It has been well documented that some E2 proteins can repress transcription of the E6 and E7 genes in HPV-transformed cells and thereby induce apoptosis (9, 11) and growth arrest/senescence (17, 18, 23, 24). However, we have shown that the LR-HPV E2 proteins can prevent HR-HPV16 E2-induced apoptosis in NIH 3T3 cells, a non-HPV-transformed cell line. Thus, it seems more likely that the LR-HPV6 and LR-HPV11 E2 proteins might block the binding of HR-HPV16 E2 to cellular DNA sequences, or cellular proteins, that are required for the induction of apoptosis. One candidate for a cellular protein that could mediate the effects of the HR-HPV16 E2 and also be a target for the LR-HPV E2 proteins is p53. Although the LR-HPV E2 proteins bind very poorly to p53 and do not induce apoptosis, at high concentrations, these proteins might be able to block HPV16 E2-induced apoptosis by preventing the HR-HPV16 E2-p53 interaction. Since the E2 proteins are dimeric and well conserved, another possibility is that the E2 proteins from LR-HPV types can form heterodimers (3) with the HR-HPV16 E2 protein and that these heterodimers are unable to induce cell death.

The role of p53 in E2-induced apoptosis has been the subject of some debate. We have shown that HR-HPV16 E2-induced apoptosis is blocked by overexpression of the HPV16 E6 gene product, a protein that is known to bind p53 and target this protein for proteolytic degradation (45). Massimi and coworkers have shown that the HR-HPV18 E2 protein binds to p53 in vitro in GST pull-down experiments and in immunoprecipitation assays (32). In addition, the HR-HPV18 E2 protein has been shown to bind CBP, a coactivator protein that is thought to be important in at least some forms of p53-dependent apoptosis (1, 37, 47). Our experiments have shown that the HR-HPV16 E2 protein can induce apoptosis in p53-null Saos-2 cells, but only in the presence of coexpressed p53. In contrast, recent work has shown that a fusion between the HR-HPV18 E2 protein and GFP can induce apoptosis in Saos-2 cells in the absence of p53 (10). Thus, it would seem likely that the HR-HPV18 E2 protein at least can induce apoptosis via p53-dependent and p53-independent pathways.

Further evidence to suggest that HR-HPV18 E2-induced apoptosis might occur via a p53-independent pathway has come from the study of HR-HPV18 E2 mutants. Mutations in the HR-HPV18 E2 protein that replace either a glutamic acid residue at position 43 or an isoleucine at position 177 with alanine result in E2 variants that induce equivalent levels of

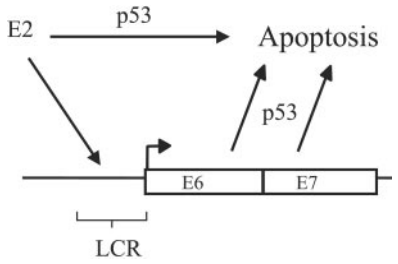


FIG. 7. HR-HPV16 E2 induces apoptosis via two pathways. (A) Model explaining the effects of HR-HPV16 E2 on the survival of HPV-transformed and non-HPV-transformed cells. HR-HPV16 E2 binds to the HPV long control region (LCR) regulates transcription of E6 and E7, and thereby induces apoptosis. In addition, this E2 protein can bind to p53 and induce apoptosis independently of E6 and E7. Mutations that inhibit the binding of E2 to p53 restrict apoptosis to HPV-transformed cells. Note that apoptosis is not the only outcome of E2 expression in HPV-transformed cells; the bovine papillomavirus E2 protein represses transcription of E6 and E7 and induces cell senescence (18, 23).

apoptosis in HeLa cells but which induce only 40% and 20%, respectively, of the mdm2 promoter activity induced by wild-type E2 (12). However, the relationship between the ability of p53 to regulate promoter activity and its ability to induce apoptosis is somewhat unclear. While some studies have concluded that transcription activation by p53 is required for p53-mediated apoptosis, other studies have concluded that transcription activation and the induction of apoptosis are separable functions. For example, p53 175P is a p53 mutant that is unable to induce apoptosis but which appears to activate transcription and induce cell cycle arrest as well as the wild-type p53 protein (39). Here we have shown that the ability of p53 to regulate transcription is not required for HR-HPV16 E2-induced apoptosis. HR-HPV16 E2 thus appears to induce a transcription-independent pathway of p53-dependent apoptosis. Interestingly, caspase-8, a caspase thought to be activated during p53-induced transcription-independent apoptosis, is activated by the HR-HPV18 E2 protein (10). The N-terminal domain of the HR-HPV18 E2 protein is sufficient to induce apoptosis via this pathway (10). Our experiments suggest that this may not the case for the HR-HPV16 E2 protein.

The data described above might help to explain why HR-HPV types are often found integrated into the host genome in cervical cancer. Presumably, in cells infected with LR-HPV types, random events that disrupt the E2 gene do not remove a proapoptotic signal, and therefore, these integration events give no selective advantage to cells that contain the integrated DNA. However, in cells infected with HR-HPV types, random events that disrupt the E2 gene remove a proapoptotic signal and give a selective advantage to the cells that contain integrated DNA. Thus, in cells infected with HR-HPV types, E2 appears to function as a tumor suppressor protein. Thus, loss of the E2 protein results in the deregulated expression of E6 and E7 and also upsets a delicate balance of proapoptotic signals from E7 and E2 and antiapoptotic signals from E6.

Finally, it is worth noting that cervical cancer is the second most common cause of cancer-related death in women and that new approaches to the treatment of this disease would be of great potential benefit. However, none of the current treatments for cervical cancer or its precancerous lesions specifically attack HPV-containing cells. Here we have shown that the HR-HPV16 E2 protein induces apoptosis in HPV-transformed cells via two pathways; E2 can induce apoptosis indirectly, via its effects on the expression of E6 and E7, and directly, via its interaction with p53 (Fig. 7). Mutations that disrupt the interaction of E2 with p53 prevent E2-induced apoptosis in non-HPV-transformed cells but have no effect on the ability of E2 to induce apoptosis in HPV-transformed cells. Mutants of E2 that only induce apoptosis in HPV-transformed cells may be efficacious in the treatment of cervical cancer and other HPV-related diseases. However, further work will be required to develop vectors that can deliver these proteins to HPV-transformed cells in vivo.

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