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Nucleo-cytoplasmic Shuttling of High Risk Human *Papillomavirus* E2 Proteins Induces Apoptosis*

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Human *Papillomavirus* (HPV) E2 proteins are the major viral regulators of transcription and replication during the viral life cycle. In addition to these conserved functions, we show that E2 proteins from high risk HPV types 16 and 18, which are associated with cervical cancer, can induce apoptosis. In contrast, E2 proteins from low risk HPV types 6 and 11, which are associated with benign lesions, do not cause cell death. We show that the ability to induce apoptosis is linked to the intracellular localization of the respective E2 proteins rather than to inherent properties of the proteins. Although low risk HPV E2 proteins remain strictly nuclear, high risk HPV E2 proteins are present in both the nucleus and the cytoplasm of expressing cells due to exportin-1 receptor (CRM1)-dependent nucleo-cytoplasmic shuttling. Induction of apoptosis is caused by accumulation of E2 in the cytoplasm and involves caspase 8 activation. We speculate that disruption of the E2 gene during viral genome integration in cervical carcinoma provides a means to avoid E2-induced apoptosis and allow initiation of carcinogenesis.

Human papillomaviruses are the etiologic agents of cervical cancer, and they are found associated with more than 90% of this type of cancer. Viruses infecting the anogenital tract can be divided into two distinct classes that are associated either with benign lesions (such as HPV[4] types 6 and 11) or with cancer (such as HPV types 16 and 18). The class of high risk HPVs associated with cervical carcinoma expresses two oncogenes, E6 and E7, which transform the cells by negatively interfering with the p53 and pRB pathways (1, 2). In contrast, the E6 and E7 proteins of low risk HPVs have been shown to be unable to induce cellular transformation *in vitro* (for review, see Ref. 3).

The HPV E2 protein plays a crucial role in the viral life cycle. E2 proteins are transcription factors that can repress transcription of the E6 and E7 genes of the genital papillomaviruses independently of their oncogenic potential (4–8). Transcriptional repression was shown to result from binding of E2 to sequences within the E6 and E7 early promoters of high risk as well as low risk HPVs (5, 9–12). Other properties of the E2 proteins are also conserved, such as their ability to activate transcription of heterologous promoters containing at least two E2 binding sites in a specific configuration and to activate viral DNA replication in concert with the E1 helicase (13). Comparative analyses of E2 proteins of high risk and low risk HPV viruses suggested a high degree of conservation in their three-dimensional structure, compatible with their conserved functions in viral DNA replication and transcription (14–17).

E2 proteins have also been shown to directly influence host cell biology. In particular, proapoptotic activity has been demonstrated for the HPV18 E2 protein (18, 19) as well as the HPV16 E2 protein (20) and was shown to be an intrinsic property independent of transcription (18, 19). More recently, an interaction between E2 and mitotic chromosomes was described through direct interaction with Brd4, a bromodomain protein associated with acetylated chromatin (21). E2 is also associated with the mitotic spindles via tubulin interactions, which was proposed to take part in the segregation of viral genomes during cell division (22). Finally, we also recently described a novel interaction of high risk HPV E2 proteins with activators of the mitotic ubiquitin ligase anaphase-promoting complex (APC/C) which, by inhibiting the degradation of various APC/C substrates, results in a strong genomic instability (23). These additional functions of the E2 proteins are mediated by direct interactions with cellular proteins rather than through transcriptional control and were shown to be independent of other viral functions.

The E2 proteins are composed of three distinct domains that can be expressed separately and retain their function (24). The carboxyl-terminal domain forms a small β-barrel dimer which binds to specific recognition sequences on the DNA (25). A hinge domain, with no specific structure, mainly serves as a flexible linker between the two functional domains. The amino-terminal domain is composed of two sub-domains separated by a fulcrum; one contains three α-helices (at its amino-terminal end), and the other is composed of a succession of β-sheets (at its carboxyl-terminal end) (15, 16). The amino-terminal domain retains functions of E2 involved in regulating transcription and replication. Above all, it has been shown to interact with cellular proteins involved in transactivation as well as with the E1 helicase that activates viral DNA replication (15, 26) and with Brd4 involved in episomal segregation during mitosis (21). Other characteristics, such as ubiquitination and degradation by the proteasome (24) as well as induction of apoptosis (19) involve this domain. The proapoptotic property of HPV18 E2 has been shown to depend on induction of the extrinsic pathway through activation of caspase 8 (19). The exact mechanism of this activation is not yet understood, but it appears that E2 activates caspase 8 through direct protein-protein interaction.[5] Induction of apoptosis has also been shown to occur with the HPV16 E2 protein, although the mechanism is not fully understood (20).

In the present work we sought to examine whether some of the autonomous properties of the HPV E2 proteins could differ among virus types, as was shown before for the E6 and E7 proteins. We examined whether the proapoptotic property of the HPV18 E2 protein is conserved among E2 proteins of other genital HPV subtypes. We fused GFP

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[4] The abbreviations used are: HPV, human *Papillomavirus*; GFP, green fluorescent protein; ORF, open reading frame; NES, nuclear export sequence; Z-VAD, benzyloxycarbonyl-Val-Ala-Asp; PBS, phosphate-buffered saline; NLS, nuclear localization signals; sub-2N, less than 2N chromosomes.

[5] C. Demeret and F. Thierry, unpublished data.

at the amino terminus of the HPV16, HPV11, and HPV6 E2 proteins and used recombinant adenoviruses to express them as previously described for HPV18 E2 (19). Subcellular localization of the E2 proteins appeared strikingly different; the E2 proteins from low risk viruses HPV6 and HPV11 were exclusively nuclear, whereas proteins from high risk viruses were located in both the nucleus and in the cytoplasm. We show that HPV18 E2 protein actively shuttles between the nucleus and the cytoplasm of the infected cells and that partial cytoplasmic localization of high risk E2 proteins correlates with induction of apoptosis through caspase 8 activation. In contrast E2 proteins of low risk HPVs do not induce apoptosis due to their exclusive nuclear localization. These experiments point to important differences in the biological functions of the E2 proteins from high and low risk papillomaviruses infecting the anogenital tract.

MATERIALS AND METHODS

Construction of Plasmids and Recombinant Adenoviruses—E2 ORFs of wild-type HPV16, HPV6, and HPV11 viruses and the different mutants were cloned in the pEGFP-C1 expression plasmid (Clontech) as previously done for the HPV18 E2 ORF (24). The E2 open reading frames of HPV6 and -11 were cloned by PCR from PCR3-E2 (a kind gift of J. Archambault) in-frame with the carboxyl terminus of GFP. Expression cassettes of the various GFP fusion proteins were introduced in place of the E1A-E1B region in replication-deficient recombinant adenoviruses by a two-step procedure in bacteria as described previously (19, 27). Recombinant adenoviruses were produced and amplified in 293 cells. Viruses were subsequently purified on CsCl gradients and titrated. Adenovirus expressing the GFP alone was purchased from Quantum. Conventional PCR-based mutagenesis was used to introduce point mutations AT to RK at position 246 and 247 and an insertion of an arginine at position 250 of the amino acids sequence of the HPV18 E2 ORF as well as mutations KR to TH at position 107 and 108 and RK to AT at position 238 and 239 of the amino acid sequence of the HPV11 E2 ORF.

The deleted mutant GFP-E2 *del 26* was prepared by exchanging the XhoI-StuI fragment digested from the pXLGFP-E2 previously described (19) by an XhoI-StuI fragment prepared from a PCR amplification with a specific primer containing an XhoI site at the 5′ end, 5′-AAAA**CTCGAG**CTAGTAAAGACATAGACAGC-3′, and a 3′ primer, 5′-CGCGGATCCACTGCACATAGAGTCATTAC-3′. The deleted mutant GFP-E2 *del53* was cloned from PCR amplification from position 53 of the amino acid sequence to the end of the HPV18 E2 open reading frame, with specific primers containing EcoRI and BamHI restriction sites for cloning in-frame with the carboxyl terminus of the GFP: 5′-C**GAATTC**CGGCATACAGACATTAAACCAC-3′ and 5′-AAA**GGATCC**TTACATTGTCATGTATCCC-3′ (restriction site are indicated in boldface).

The putative nuclear export sequence (NES) was amplified by PCR from HPV18 E2 with specific primers containing the BamHI and XbaI restriction sites for cloning in-frame with the carboxyl terminus of the GFP nuclear localization signals (NLS) expression plasmid previously described (24). The primers used were 5′-CGC**GGATCC**GACATAGACAGCCAAATACAG-3′ and 5′-CTAG**TCTAGA**TTAAAAGAATATTGCATTTTCCC-3′.

Cell Cultures, Transient Transfections, and Infections—MCF7 and HeLa cells were grown in 6- or 10-cm Petri dishes in Dulbecco's modified Eagle's medium supplemented with 7% fetal calf serum. Saos-2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transient transfections of HeLa cells were done by the standard calcium phosphate co-precipitation technique, as previously described (5), with 5 or 10 μg of the GFP expression plasmids in 6-cm Petri dishes.

Infections were done at multiplicity of infection (m.o.i.) varying from 200 to 1000 with the various recombinant adenoviruses. Cells were incubated with 1 ml of Dulbecco's modified Eagle's medium without serum containing the adenovirus dilutions complemented with 4 μM Polybrene for Saos-2 and MCF7 cells for 1 h at 37 °C.

When indicated, cells were treated with the pan caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK) (40 μM, Biomol) or an equivalent volume of Me_2SO, added in the medium just after infection. 24 h post-infection cells were treated with 10 ng/ml leptomycin B (Sigma). Inhibition of the proteasome was obtained by treating the cells with 10 μM lactacystin (Calbiochem) for 6 h.

For video microscopy, treated cells were transferred in a thermostatted chamber (7% CO_2 at 37 °C) under the objective of the microscope, where they were maintained for 4 h. Images were captured every 5 min in phase contrast and in fluorescence using a 20× objective on an Axiovert Zeiss microscope. Images were processed by the Metamorph software and converted to Adobe Photoshop.

Immunofluorescence—HeLa cells grown on cover slips were rinsed with phosphate-buffered saline (PBS) 24 h after infection or transfection and then fixed in 2% paraformaldehyde/PBS for 1 h at 4 °C. After rehydration, cells were permeabilized and saturated with PBS containing 2% serum and 0.1% Triton for 30 min. They were then incubated with primary antibodies anti-p53 (Santa Cruz PAB1801), anti-HPV18 E2 (24), anti-HPV16 E2 (Ab261), anti-HPV11, and 6 E2 (kind gifts from J. Archambault) for 1 h followed by incubation with the appropriate secondary antibody coupled to Texas Red. Nuclei were stained by 4′,6′-diamidino-2-phenylindole, 0.15 μg/ml. Annexin labeling was done according to the manufacturer with annexin V-PE (BD Pharmingen). Pictures were taken with a Zeiss Axiophot microscope coupled to a CCD camera and were processed by Metaview software and converted to Adobe Photoshop.

In Vitro Caspase Cleavage—HeLa cells expressing GFP-E2 proteins were harvested, and cell lysates were prepared in p300 buffer (20 mM NaH_2PO_4, 250 mM NaCl, 30 mM $Na_4P_2O_7$·10 H_2O, 0.1% Nonidet P-40, 5 mM EDTA, 5 mM dithiothreitol, protease inhibitor (Roche Applied Science)) for 30 min at 4 °C followed by centrifugation. Protein G PLUS-agarose (Santa-Cruz sc-2002) was used to preclear the extracts. Immunoprecipitation was done with a monoclonal anti-GFP antibody (Roche Applied Science 1814460). Immune complexes were collected on protein G PLUS-agarose and washed 3 times with p300 buffer. One-tenth of the immunoprecipitate was used to perform *in vitro* cleavage by recombinant caspase 3 (BD Pharmingen 556472) according to the manufacturer's instructions. Results were visualized by Western blot with an anti-GFP antibody.

Western Blot Experiments—Cells were either resuspended directly in Laemmli sample buffer or extracted in p300 buffer (20 mM NaH_2PO_4, 250 mM NaCl, 30 mM $NaPP_i$, 0.1% Nonidet P-40, 5 mM EDTA, 5 mM dithiothreitol) for 30 min at 4 °C followed by centrifugation. Equal amounts of total cells or cellular extracts were denatured in Laemmli sample buffer, boiled, and separated by 12% SDS-PAGE acrylamide gels. After overnight transfer, nitrocellulose membranes were saturated in PBS-Tween plus 10% milk, incubated with the appropriate first antibodies anti-GFP (Torrey Pines Biolab TP401), anti-caspase 8 (BD biosciences 559932), anti-actin (Sigma A2066) for 1 h, and washed with PBS-Tween. Incubations with secondary antibodies coupled to peroxidase were done for 1 h followed by washing in PBS-Tween. Membranes

were revealed using ECL plus Western blot detection system from Amersham Biosciences.

Pulse-Chase Experiments—HeLa cells were incubated with medium deficient in methionine and cysteine and supplemented with 5% dialyzed fetal calf serum for 30 min. Radiolabeling with Tran^{35}S-label (0.25 mCi/ml; ICN) was done for 30 min followed by chase with complete medium for the indicated time points. Cell lysates were prepared in p300 buffer (20 mM NaH_2PO_4, 250 mM NaCl, 30 mM $Na_4P_2O_7$, 10 H_2O, 0.1% Nonidet P-40, 5 mM EDTA, 5 mM dithiothreitol, protease inhibitor (Roche Applied Science)). Immunoprecipitation of GFP-E2 proteins were done as for *in vitro* cleavage experiments. Proteins were eluted in SDS-PAGE sample buffer, boiled, and separated by SDS-PAGE on a 12% gel. Gels were fixed, dried, and autoradiographed. Quantification was performed using a PhosphorImager and ImageQuant software (Molecular Dynamics).

Flow Cytometry Analyses—Cells were harvested between 24 and 48 h post-infection, washed once with PBS, and fixed in ice-cold 100% ethanol. Cells were rehydrated in PBS with 2% serum. In some cases cells were treated with 0.2 M Na_2HPO_4 and 40 mM citric acid for 15 min to favor extraction of low molecular weight DNA. DNA was stained with propidium iodide (20 μg/ml) plus 10 μg/ml RNase for 30 min and analyzed with epics XL fluorescence-activated cell sorter (Coulter). For annexin detection cells were harvested in binding buffer and labeled with annexin V-PE before analysis according to the manufacturer instructions (BD Bioscience).

RESULTS

Comparison of High Risk and Low Risk HPV E2 Expression and E2-induced G_1 Arrest in HeLa Cells—We prepared recombinant adenoviruses expressing four different amino-terminal GFP-E2 fusion proteins originating from human genital papillomaviruses of either high risk (16 and 18) or of low risk (6 and 11) types. We infected HeLa cells at comparable m.o.i. to get GFP fluorescence in virtually all the cells present on the plates. As expected, GFP was observed in the nuclei of cells infected by the four recombinant adenoviruses expressing E2; however, cytoplasmic fluorescence was also clearly visible for the high risk HPV16 and HPV18 E2 (Fig. 1*A*). Western blots analysis indicated that differences in the subcellular localization did not depend on differences in the levels of expression. Notably, cytoplasmic localization of high risk HPV E2 proteins was not necessarily associated with higher levels of protein expression as shown with the HPV16 E2 protein (Fig. 1*B*). In contrast, even when expressed to high levels, low risk HPV11 and HPV6 E2 proteins remain strictly nuclear (Fig. 1*A* and not shown). Because HPV18 E2 has been shown to be particularly unstable (24), we compared the stability of the four E2 proteins in pulse-chase experiments (Fig. 1*C*). These experiments showed that the HPV16 and HPV18 E2 proteins exhibit a similar half-life of 50 min, which exactly matches the half-life reported previously for HPV18 E2 (24). In contrast the HPV6 and HPV11 E2 proteins appeared more stable, although their half-life times were only increased by 1.5-fold for HPV11 E2 (75 min) and by 2.5-fold for HPV6 E2 (125 min). We concluded from these experiments that the exclusive nuclear localization of E2 proteins of the low risk viruses did not correlate with strong stabilization of the proteins.

We also checked whether all the E2 proteins were able to repress transcription of the viral oncogenes in HeLa cells, as previously reported (28–30). It has been shown that this transcriptional repression induces p53 stabilization in HeLa cells due to E6 repression, consequently inducing a strong G_1 growth arrest (28, 31). The presence of p53 in cells expressing E2, but not in control cells, clearly indicated that the four proteins were equally able to stabilize nuclear p53 in infected cells (Fig. 1*A*) and to induce G_1 growth arrest (Fig. 1*D*). We also checked the transactivation and DNA binding properties of the four proteins and detected no significant differences (data not shown). These experiments show that the GFP fusion proteins retain biological activity of the native proteins, as reported for the HPV18 E2 (24).

High Risk HPV E2 Proteins Induce Apoptosis—Because the four GFP-E2 fusion proteins appear to maintain their biological activity, we decided to compare their proapoptotic activities by infecting HeLa cells at increasing m.o.i. with the respective recombinant adenoviruses. We previously showed that induction of apoptosis requires a threshold expression level of the HPV18 E2 protein (19). Indeed, apoptosis was clearly detectable in cells infected at higher m.o.i. of HPV18 and HPV16 GFP E2-expressing recombinant adenoviruses, as shown by direct microscopic observation (*black arrows*, Fig. 2*A*). Under similar conditions, cells infected with recombinant adenoviruses expressing HPV6 and HPV11 E2 proteins did not die, even at high m.o.i., correlating with accumulation of fluorescence in the nuclei of infected cells (Fig. 2*A*). The infected cells were subjected to flow cytometry, which detected the presence of sub-2N populations of up to 70% at the highest m.o.i. of HPV18 and HPV16 E2-expressing cells, in contrast to HPV6 and HPV11 E2-expressing cells, where this population remained under 10% (Fig. 2*B*). Accumulation of the E2 proteins in infected cells was confirmed by Western blot analysis (Fig. 2*C*). These experiments indicated that the full-length HPV18 and HPV16 E2 proteins did not accumulate to high levels, partly due to the emergence of specific cleavage products and degradation with increasing m.o.i. (Fig. 2*C*). The cleavage products were inhibited by the pan-caspase inhibitor Z-VAD *in vivo* (Fig. 2*D*) and result from caspase 3 cleavages of the HPV18 and HPV16 GFP fusion proteins at cryptic sites, as shown by *in vitro* cleavage experiments (Fig. 2*D*). These cleaved products are, thus, indicators of caspase activation and apoptosis in infected cells. In contrast, full-length HPV6 and HPV11 E2 proteins accumulated to levels that were higher than the two other E2 proteins due to the absence of caspase cleavage (Fig. 2*D*) and better stability.

We checked whether stabilization of the GFP-E2 proteins of HPV18 or HPV11 would change the levels of apoptosis (Fig. 2*E*). Inhibition of the proteasome by lactacystin induces an accumulation of the GFP-E2 proteins as shown in the lower panel of Fig. 2*E* but did not modulate apoptosis (*upper panel*, Fig. 2*E*). Thus, we can conclude from this experiment that the stabilization of E2 does not seem to change the apoptotic phenotype whether the E2 protein is proapoptotic, like high risk HPV18 E2, or not, like low risk HPV11 E2 protein.

High Risk HPV E2 Proteins Induce Apoptosis via a Common Mechanism Involving Caspase 8 Activation in Different Cell Lines—Clear differences in the behavior of the E2 proteins from high risk HPVs compared with low risk HPVs in inducing apoptosis was also demonstrated in other cell lines such as breast cancer MCF7 cells, containing a wild-type p53, and osteosarcoma Saos cells, which lack p53 (Fig. 3*A*). We deduced from these experiments that the E2 proteins from low risk viruses are unable to induce high levels of apoptosis in conditions where E2 proteins from high risk viruses were efficient apoptotic inducers.

We tested whether the pan caspase inhibitor Z-VAD could inhibit HPV16 as well as HPV18 E2-induced apoptosis as demonstrated before (19). The addition of Z-VAD totally abolished the appearance of a sub-2N population (Fig. 3*B*). These results suggested that the two viral proteins induced apoptosis through caspase 8 activation, as previously shown for HPV18 E2 (19). In living cells, caspase 8 exists as a full-length "proform" protein of 50/55 kDa (doublet). This form of caspase is inactive and is activated by autocleavage to give rise to the active heterodimers, able to induce apoptosis. We examined cleaved products of

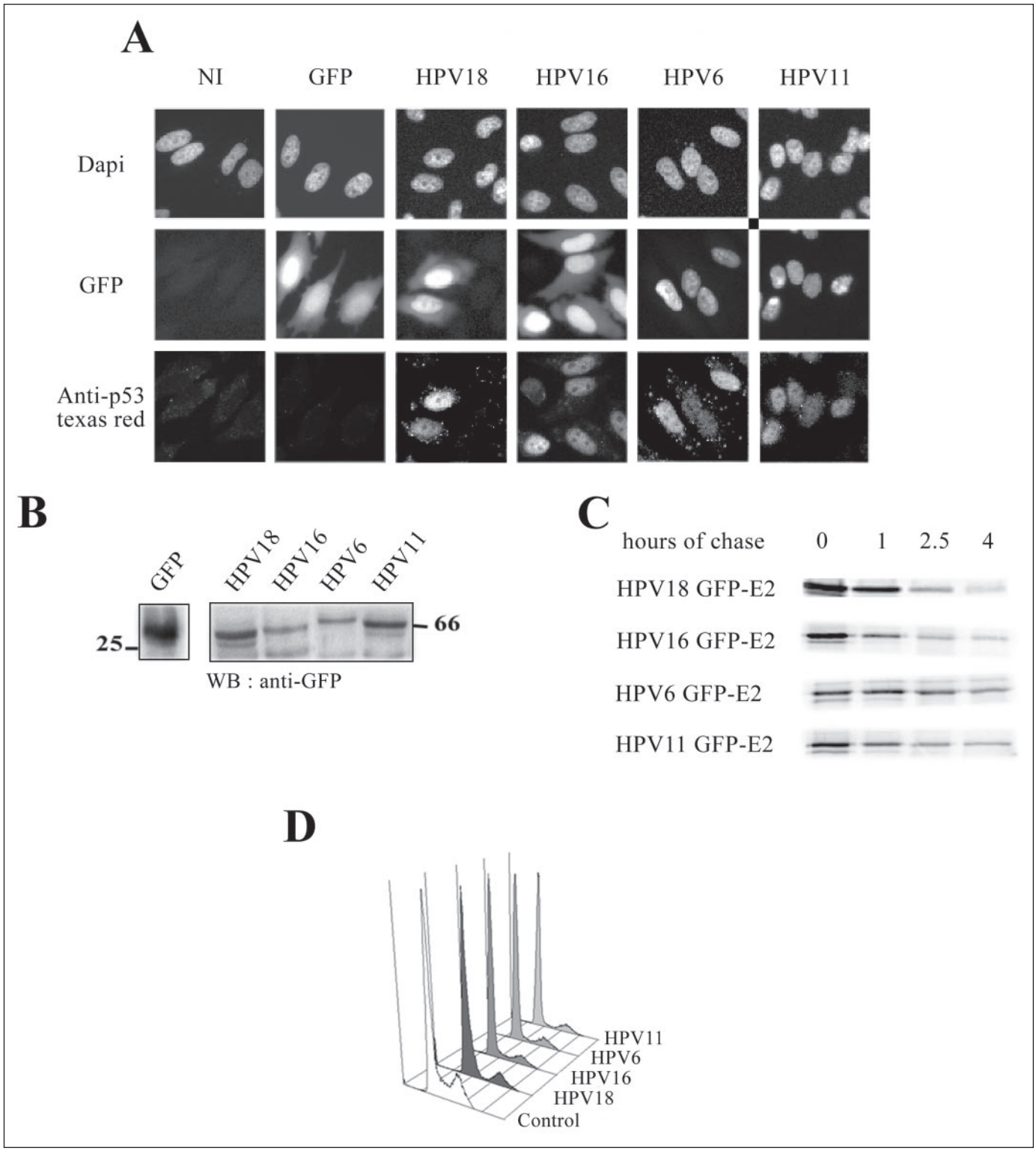

FIGURE 1. **Characterization of the recombinant adenoviruses.** *A*, stabilization of p53 by E2 proteins from HPV types 18, 16, 6, and 11. HeLa cells were either non-infected (*NI*) or infected at m.o.i. 500 with recombinant adenoviruses expressing GFP and the four GFP-E2 as indicated. Direct GFP fluorescence as well as detection of p53 by immunofluorescence using an anti-p53 antibody followed by Texas Red-coupled secondary antibodies is shown. *Dapi*, 4′,6′-diamidino-2-phenylindole. *B*, levels of expression of the E2 proteins are detected by Western blots (*WB*) done on equivalent quantities of protein extracts. *C*, pulse-chase experiments done with the four E2 proteins. HeLa cells infected at m.o.i. 200 were labeled for 30 min with Tran^{35}S-labeled methionine and cysteine and chased for 4 h. At the indicated time points, cells were extracted, and equal amounts of proteins were migrated on SDS-PAGE and exposed. Radioactivity was counted, and the half-lives were calculated to be 50 min for both HPV18, as previously described (24), and HPV16 E2 proteins and 125 min for HPV6 E2 and 75 min for HPV11 E2. *D*, G_1 arrest in HeLa cells infected with the four recombinant adenoviruses expressing E2. Cells were infected at m.o.i. 250, and cell cycles were analyzed by flow cytometry 24 h post-infection.

caspase 8 in dead cells infected by recombinant adenoviruses expressing HPV18, HPV16, HPV11, and HPV6 GFP E2 proteins (Fig. 3*C*). As expected from our previous data, HPV18 E2-expressing dead cells lost almost all caspase 8 proform and exhibited an intermediate cleaved product (40/36 kDa doublet). Interestingly, exactly the same pattern was seen in HPV16 E2-expressing cells, indicating unambiguously that

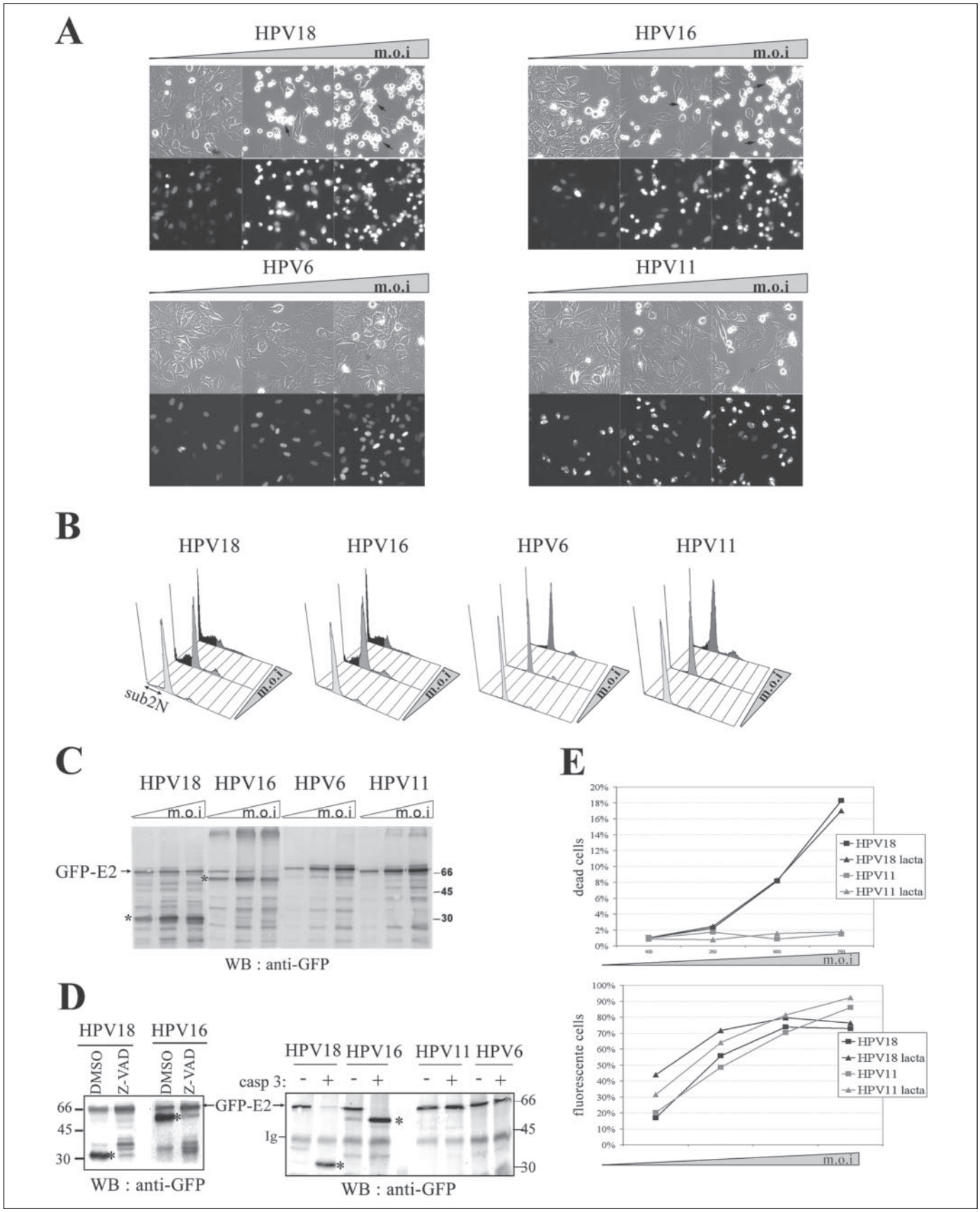

FIGURE 2. **Apoptosis is induced by high risk but not low risk viruses E2 proteins.** *A*, apoptosis in infected HeLa cells visualized by direct microscopy. HeLa cells were infected at increasing m.o.i. from 500 to 2000 plaque-forming units per cell, and images were taken 40 h after infection with an Axiovert microscope in phase contrast and fluorescence. Several dead cells are indicated by *arrows*. *B*, analyses of the sub-2N populations by flow cytometry. Cells infected in the same conditions as above were analyzed by flow cytometry, the sub-2N populations are marked in *black*. The sub-2N populations represented from 2 to 10% of the total population of cells infected with HPV6 and HPV11 E2 recombinant viruses

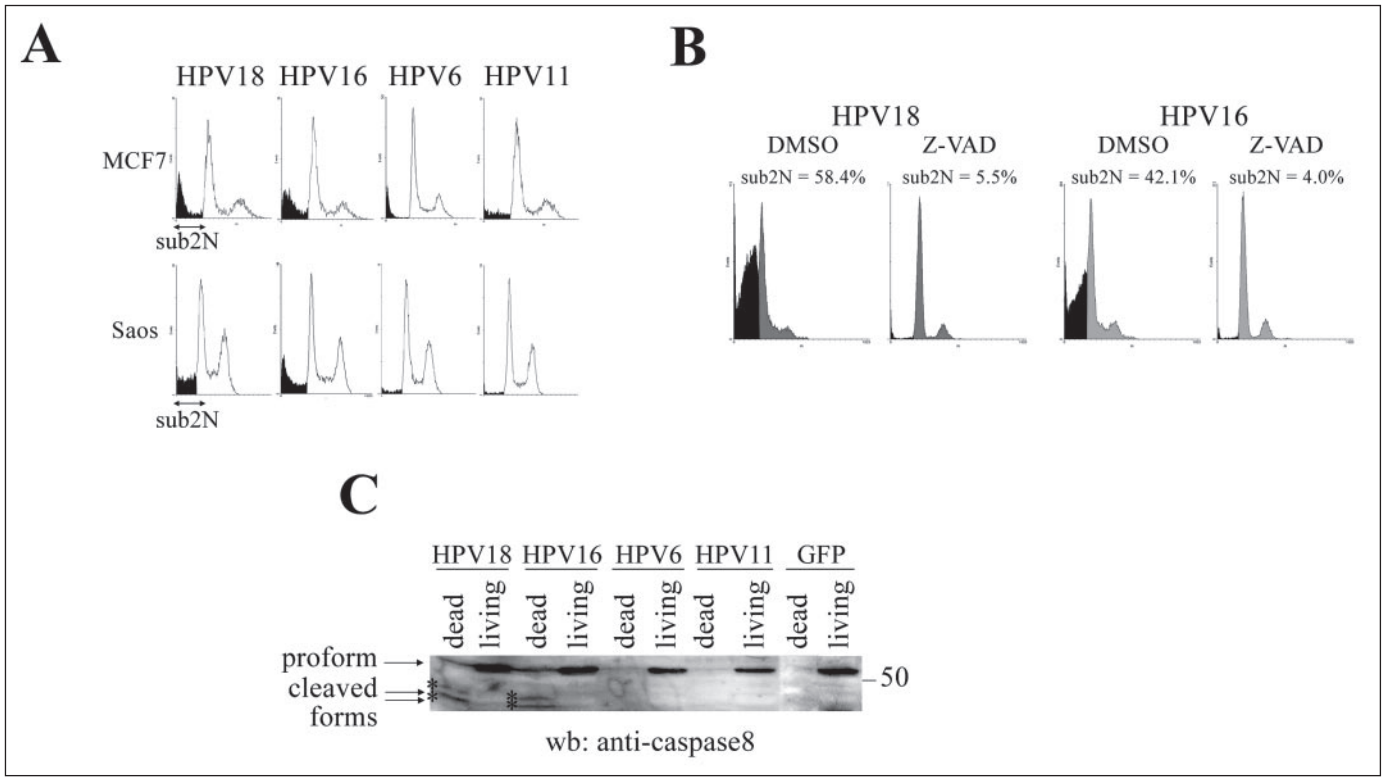

FIGURE 3. **Apoptosis by high risk viruses E2 proteins involves caspase activation through the extrinsic pathway.** *A*, high risk, but not low risk E2 proteins induce apoptosis in other cell lines. MCF7 and Saos cells, which contain, respectively, a wild-type p53 and a disrupted p53, were infected at m.o.i. 1000 with each of the four recombinant adenoviruses. The cells were subjected to flow cytometry analyses; sub-2N populations are in *black*. In MCF7, the sub-2N population corresponded to 12% of the cells infected by recombinant viruses expressing the low risk E2 proteins, whereas it represented about 25% of the population in high risk E2 recombinant adenovirus-infected cells. In the Saos cells, the sub-2N populations are 17 and 21% for HPV18 and HPV16, respectively, and 4% for both HPV6 and HPV11 E2 proteins. *DMSO*, Me_2SO. *B*, sub-2N populations disappeared after treatment with the pan-caspase inhibitor Z-VAD of HeLa cells infected by HPV18 and HPV16 E2 recombinant adenoviruses at m.o.i. 1000. *C*, detection of caspase 8 in cells expressing HPV18, HPV16, HPV6, and HPV11 E2 proteins. HeLa cells were infected at m.o.i. 750 with the HPV GFP-E2 recombinant adenoviruses, and the dead and living cells were separated (dead cells are in the supernatants) and immunoblotted (*wb*) with anti-caspase 8 antibodies. The cleaved forms of caspase 8 were detected specifically in the dead cell populations of HPV18 and HPV16 E2-infected cells.

the two high risk HPV proteins induce apoptosis through the same pathway (Fig. 3*C*). In the same experiment, no cleaved product of caspase 8 could be detected in low risk viruses GFP-E2-expressing cells.

Different NLS Regulate Subcellular Localization of the E2 Proteins from High Risk and Low Risk Viruses—As previously shown, the localization of the four GFP-E2 proteins was clearly different as detected by the GFP fluorescence. We checked whether the GFP fluorescence reflected localization of the fusion proteins by immunofluorescence using specific antibodies against each E2 protein. We observed a good superposition of the direct GFP fluorescence with Texas Red images, except for the nucleolar GFP fluorescence, indicating that E2 proteins from high risk viruses were expressed both in the nuclei and cytoplasm of infected cells, whereas HPV6 and 11 E2 proteins were exclusively nuclear (Fig. 4*A*).

NLSs have been described in the E2 protein of BPV1 (32) and HPV11 (33). Careful examination of E2 proteins sequences revealed a conserved NLS in the four HPV E2 proteins within the DNA binding domain (Fig. 4*B*). In contrast, the NLS found in the hinge of HPV11 and HPV 6 E2 proteins (33) does not seem to be conserved in the high risk HPV 18 and HPV16 E2 proteins. Another NLS in the amino-terminal domain could be found by sequence homology with BPV1 E2 (32) in HPV6 and HPV11 E2 proteins but not in HPV18 and HPV16 E2 proteins (Fig. 4*B*).

Based on these comparative sequence data, we made three different mutants of the E2 proteins to try to modify their subcellular localization. Our goal was to render the GFP HPV18 E2 exclusively nuclear and, in contrast, to make the GFP HPV11 E2 partly cytoplasmic. For this purpose, first we added a NLS in the hinge domain of the HPV18 E2 protein (18E2-*ins*NLS2) by substituting two amino acids and inserting an arginine at position 250 to mimic the HPV11 E2 hinge NLS (Fig. 4*B*). Second, we mutated the putative NLS of the amino-terminal domain of the GFP HPV11 E2 protein (11E2-*mut*NLS1) by changing two basic amino acids with the corresponding amino acids present in the HPV18 E2 protein. A third mutant was generated by changing two amino acids in the NLS of the hinge of HPV11 E2 (11E2-*mut*NLS2) (Fig. 4*B*). These mutants were transfected in HeLa cells, and their localization was checked directly in living cells by the GFP fluorescence and by immunofluorescence with specific anti-E2 antibodies. As shown in Fig. 4*A*, mutations in the amino-terminal NLS1 of the HPV11 protein did not

at increasing m.o.i., whereas they were from 6 to 70% with HPV18 and HPV16 E2 recombinant viruses. *C*, Western blot (*WB*) analysis of the proteins expressed in the infected cells presented in *panels A* and *B*. The same quantity of cells was extracted and analyzed on SDS-PAGE, then blotted and revealed by anti GFP antibody. *Stars* indicate caspase 3 cleavage products of the E2 proteins at cryptic sites of the HPV18 and HPV16 E2 proteins. *D*, the addition of the pan caspase inhibitor Z-VAD *in vivo* inhibits the appearance of the cleaved products (*left panel*). Cleavage experiments with a recombinant caspase 3 *in vitro* show that these products result from caspase 3 cleavage of high risk E2 proteins but not low risk E2 proteins (*right panel*). *E*, stabilization of the GFP-E2 proteins do not influence apoptotic induction. Saos cells infected at increasing m.o.i were treated with either Me_2SO or the proteasome inhibitor (lactacystin (*Lacta*)). Inhibition of proteasome leads to stabilization of the two E2 proteins of HPV18 and HPV11 as seen by the increase of the percentage of fluorescent cells among living cells (*lower graph*). Levels of apoptosis, deduced from the sub-2N populations in fluorescence-activated cell sorter analysis of the same samples, are not affected (*upper graph*).

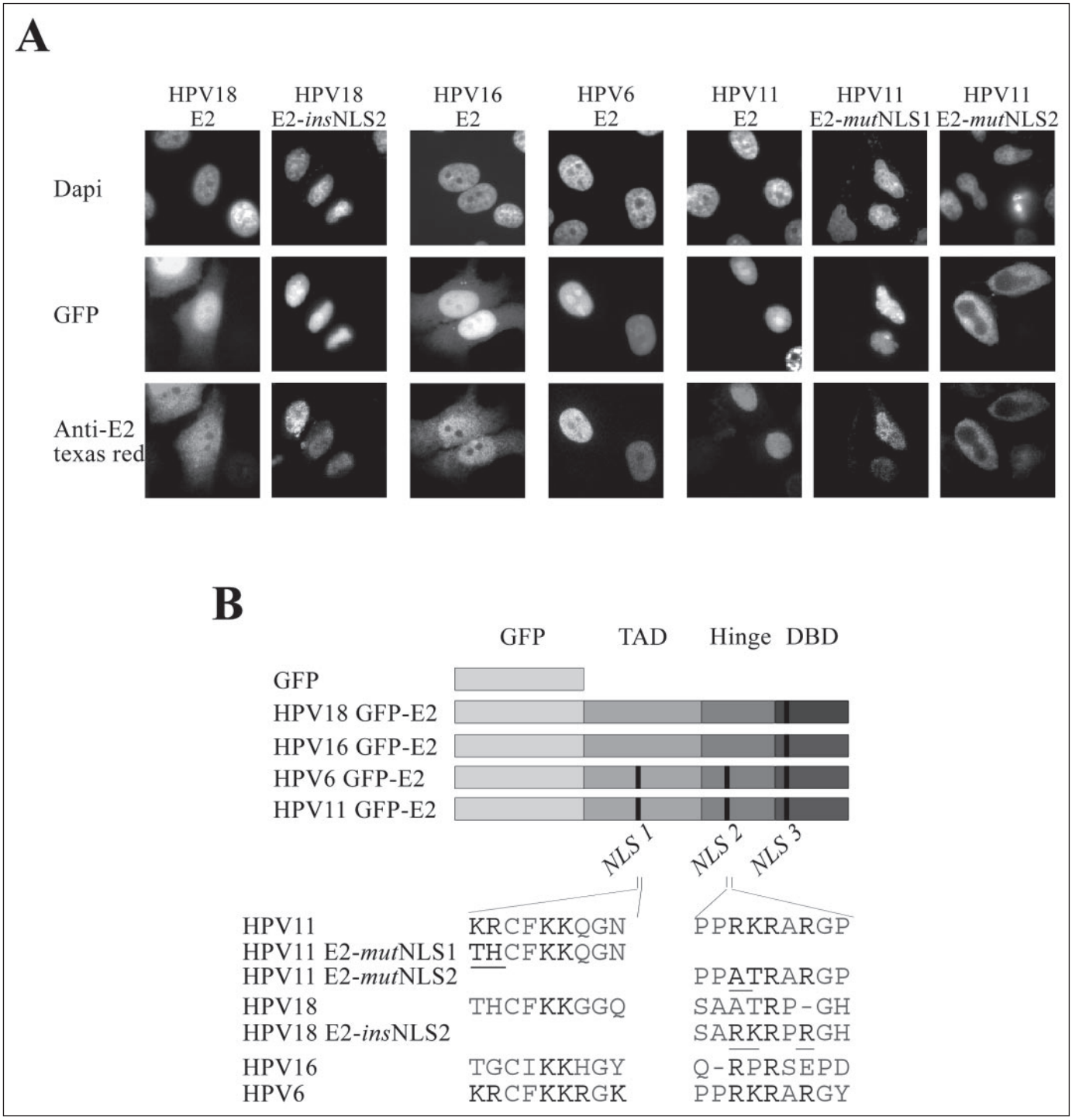

FIGURE 4. **Subcellular localization of the E2 proteins from high risk and low risk viruses are different.** *A*, high risk E2 proteins are both nuclear and cytoplasmic, whereas low risk E2 proteins are essentially nuclear. GFP fluorescence of HeLa cells infected at m.o.i. 100 with the adenoviruses expressing the wild-type GFP E2 fusion proteins is shown as well as cells transfected with the point mutants as indicated at the *top* of the *figure*. The corresponding Texas Red immunofluorescence obtained with specific anti-E2 antibodies is shown as indicated. *Dapi*, 4′,6′-diamidino-2-phenylindole. *B*, comparison of the sequences of the NLSs of the four E2 proteins. The point mutations that add a new NLS in the hinge of HPV18 E2 are shown (18-*ins*NLS2) as well as those that destroy the NLS in the amino-terminal domain of the HPV11 E2 protein (11-*mut*NLS1) and the point mutations that destroy the NLS of the hinge of the HPV11 E2 protein (11-*mut*NLS2). *DBD*, DNA binding domain. *TAD*, transactivation domain.

modify its exclusive nuclear localization, whereas in striking contrast, mutation in the NLS2 of the hinge induced nuclear exclusion as previously shown (33). The reverse experiment of adding a strong NLS in the hinge of the HPV18 E2 protein rendered this protein more nuclear as expected (Fig. 4*A*). These experiments indicated that the presence or absence of a functional NLS in the hinge domain of the HPV18 E2 protein dictate the subcellular localization of the protein.

The HPV18 E2 Protein Contains a NES in Its Amino-terminal Domain—Previous unpublished data indicated that deletion of the amino-terminal domain of HPV 18 E2 resulted in a protein with increased nuclear accumulation. These observations suggested that the HPV18 E2

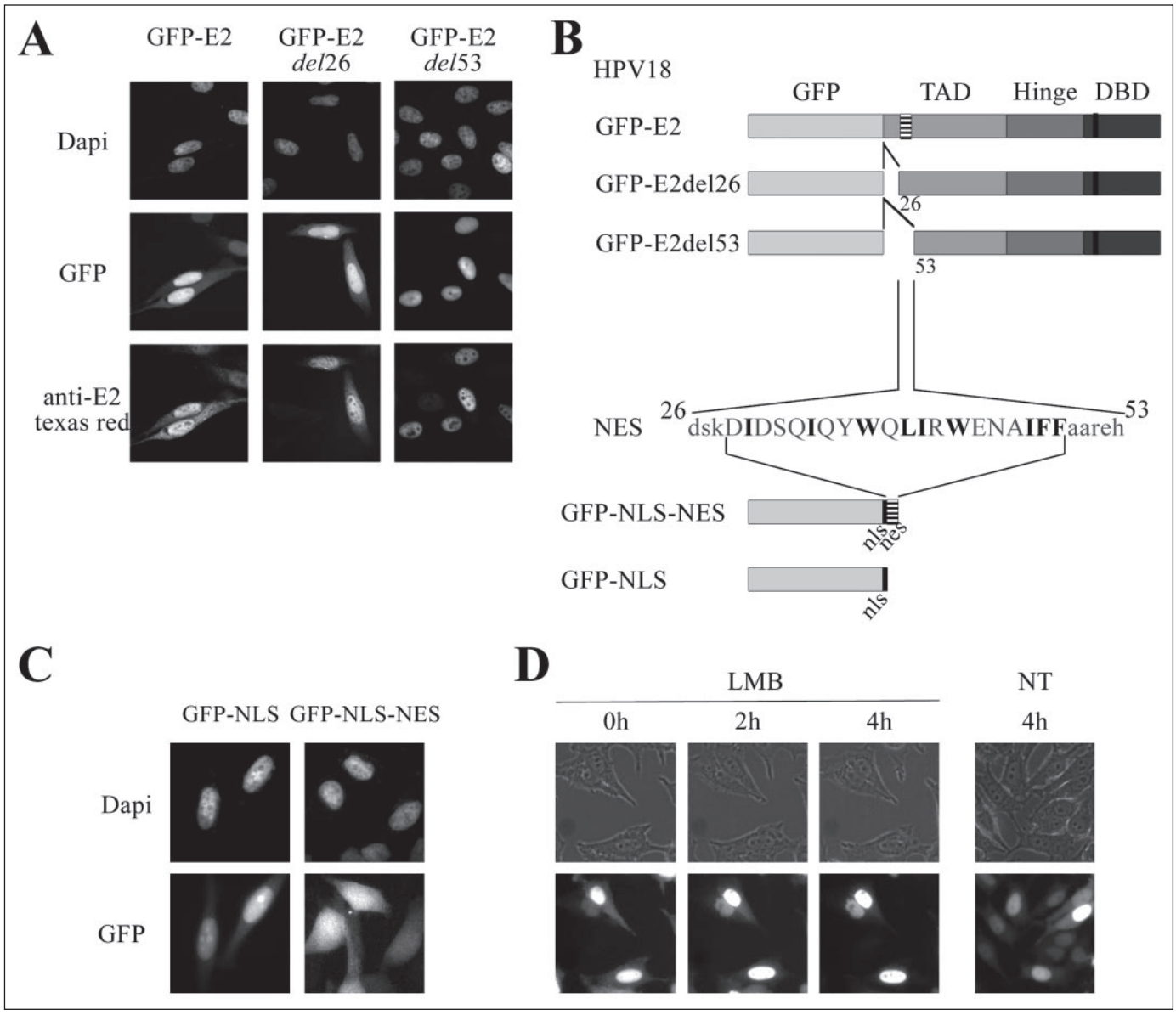

FIGURE 5. **The HPV18 E2 protein contains a NES in its amino-terminal domain.** *A*, deletion of the first amino-terminal helix (E2-*del26*) did not alter HPV18 E2 localization, whereas deletion of the first two helices (E2-*del53*) rendered it exclusively nuclear. Shown is localization of the deleted HPV18 E2 proteins by direct GFP fluorescence, (*upper panels*) and by immunofluorescence with specific anti-E2 antibodies (*lower panels*). *Dapi*, 4′,6′-diamidino-2-phenylindole. *B*, the second α-helix contains a putative NES sequence characterized by a sequential incidence of large hydrophobic amino acids. *DBD*, DNA binding domain. *TAD*, transactivation domain. *C*, sequences between amino acids 29 and 47 are sufficient to delocalize a GFP-NLS fusion protein to the cytoplasm. HeLa cells were transfected with the two plasmids expressing the fusion proteins GFP-NLS or GFP-NLS-NES as indicated. Direct GFP fluorescence is shown as well as 4′,6′-diamidino-2-phenylindole staining of the nuclei. *D*, cytoplasmic localization of E2 is due to a CRM1-dependent shuttling. HeLa cells infected with the recombinant adenovirus expressing HPV18 GFP-E2 were treated with leptomycin B (*LMB*) 24 h post-infection and were subjected to video microscopy. *NT*, non-treated cells.

protein could possess a NES in its amino-terminal domain (34, 35). To address this, we generated deletions of the amino-terminal domain of the HPV18 E2 protein and fused the resultant polypeptides to GFP to study subcellular localization. The E2-*del53*, lacking the first 53 amino-acids corresponding to the two first α-helices, according to the published crystal structure (15, 16), clearly localizes differently from the full-length E2 protein. E2-*del53* accumulates to high levels in the nucleus, displaying localization similar to the low risk HPV E2 proteins or HPV18 E2 protein lacking the entire amino-terminal domain. Interestingly, deletion of only one α-helix of the amino-terminal domain (E2-*del26*) had an extensive cytoplasmic localization similar to or even greater than the full-length protein (Fig. 5*A*). We, thus, speculated that a NES could lie within the second α-helix between residues 26 and 53 of the HPV18 E2 protein. Examining the sequences in this region, we found a cluster of about 20 amino acids that contains a putative consensus NES sequence (35–37) (Fig. 5*B*).

We fused the potential E2-NES to GFP containing the NLS of the HPV18 E2 DNA binding domain (24). The addition of the putative NES sequence caused a strong delocalization of the nuclear GFP to the cytoplasm of transfected cells (Fig. 5*C*), clearly indicating that this sequence is sufficient to promote nuclear export of a fused protein. To further investigate the process of E2 nuclear export, we used leptomycin B, an inhibitor of CRM1, which is one of the main exportins responsible for protein export from the nucleus to the cytoplasm. By time lapse microscopy, we found that the GFP-E2 18 protein accumulated in the nuclei of infected cells and almost completely disappeared from the cytoplasm after 4 h of treatment with leptomycin B (Fig. 5*D*). These experiments strongly suggest that the HPV18 E2 is able to shuttle from the nucleus to the cytoplasm via CRM1-depend-

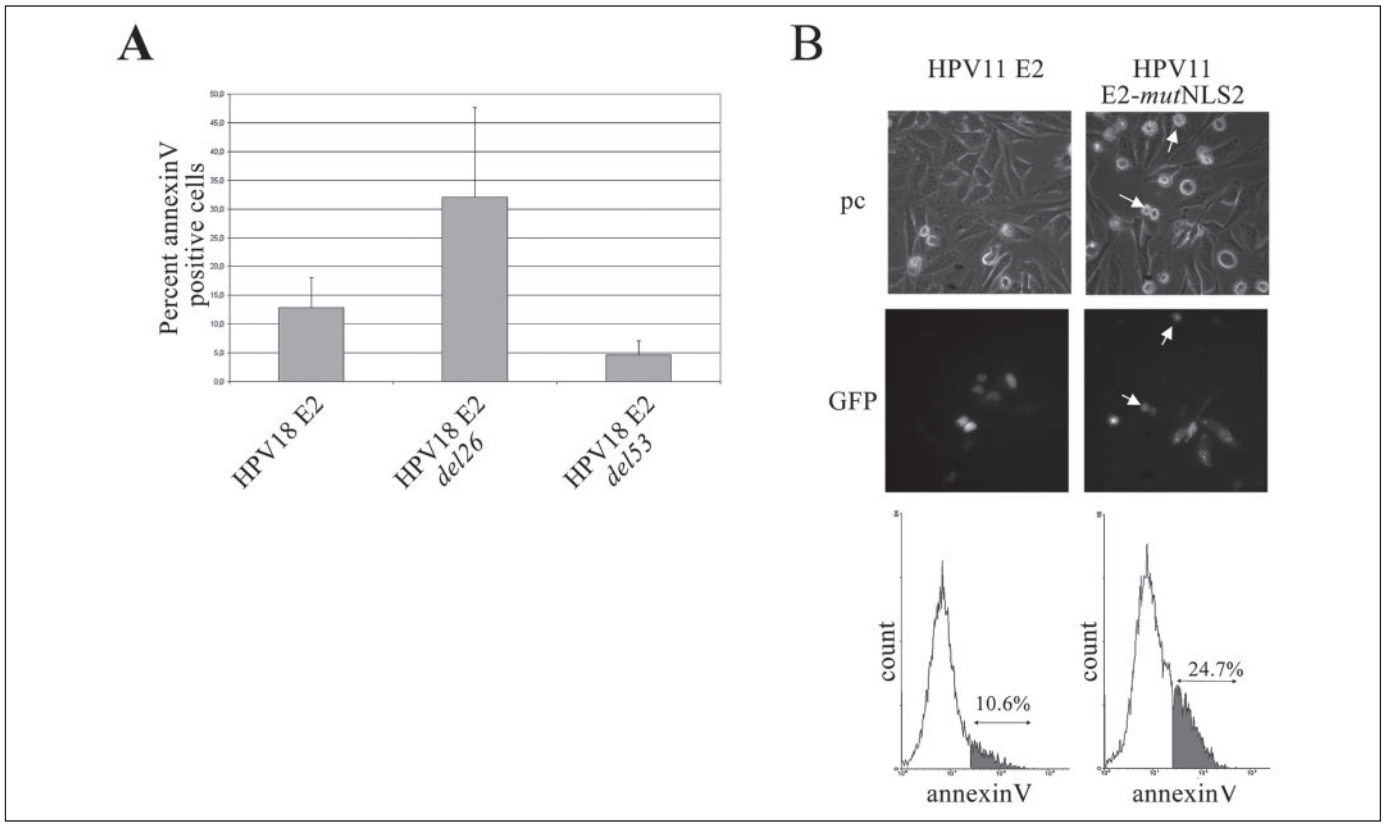

FIGURE 6. **Subcellular localization of E2 determines the apoptotic phenotype.** *A*, HeLa cells were transfected with the HPV18 full-length and two deleted GFP-E2 fused proteins as indicated. Green fluorescent cells, which were labeled by annexin V coupled to phycoerythrin were counted and reported on the graph. *B*, HeLa cells transfected with the HPV11 GFP-E2 proteins, either wild type or mutated, in the NLS are visualized in phase contrast (*pc*) and fluorescence (*upper panels*); dead cells are indicated by *arrows*. The cells labeled by annexin V were quantified by fluorescence-activated cell sorter analysis (*lower panels*).

ent mechanism. Thus, subcellular localization of E2 proteins seems to result from a complex interplay between signals of nuclear import and export.

Subcellular Localization of E2 Correlated with Its Proapoptotic Activity—Apoptosis with the high risk HPV E2 proteins coincides with the presence of E2 proteins in the cytoplasm. We, thus, examined the apoptotic properties of the different E2 mutants that vary in their subcellular localization. The two amino-terminal deleted HPV18 E2 proteins strikingly differ in their proapoptotic abilities; E2-*del53*, which is exclusively nuclear, did not induce apoptosis above background, whereas E2-*del26*, which is partly cytoplasmic, was a strong inducer of apoptosis (Fig. 6*A*). These results led us to examine the behavior of the NLS point mutants described above. We examined the level of apoptosis induced by the HPV18 E2 mutant that has been rendered more nuclear by the addition of the hinge NLS of HPV11 E2. Our experiments indicated an intermediate phenotype between wild-type E2 and the amino-terminal deleted E2 protein that was difficult to quantify precisely in transfection experiments. In contrast, the cytoplasmic NLS mutant HPV11 E2 protein could induce apoptosis, as measured by direct observation and annexin labeling, to higher levels compared with the HPV11 E2 wild-type protein (Fig. 6*B*). We deduced from these experiments that differences in the induction of apoptosis between the two types of E2 proteins were not due to inherent differences in effector functions but, rather, to their different subcellular localization.

DISCUSSION

The roles of *Papillomavirus* E2 proteins in the viral life cycle have been studied extensively. Human *Papillomavirus* E2 proteins exhibit a transactivation function whose role is not yet understood in the course of the viral life cycle. In contrast, E2 function as a transcriptional repressor of the viral oncogenes and the specific disruption of the E2 gene during the establishment of HPV-induced cervical carcinogenesis have been well documented. E2 also activates viral DNA replication by interacting cooperatively with the viral helicase E1 at the origin of replication in the viral genome. Apart from its functions in regulating transcription and viral DNA replication, E2 proteins also appear to be able to interact physically with cellular proteins to directly influence host cell biology. One of these functions, not linked to transactivation, is the induction of apoptosis, which has been shown to occur through induction of the extrinsic death pathway and activation of caspase 8 (19). We have also shown that this apoptosis is independent of p53 and can occur in many different cell types provided that the viral protein is expressed above a certain threshold. This activity has also been described for the HPV16 E2 protein, although it was reported that induction of apoptosis required p53 activation (20). Nothing was known about the propapoptotic functions of other E2 proteins, particularly for the E2 proteins from other human papillomaviruses involved in benign infections of the anogenital tract such as the low risk HPV types 6 and 11.

One of the most notable differences between the E2 proteins of the two types of HPV viruses was their subcellular localization. Although the HPV11 and HPV6 E2 proteins were exclusively nuclear in many cell types tested, the HPV16 and HPV18 proteins were also found in the cytoplasm. We could show that these differences in behavior were due to the fact that a dominant functional NLS was present in the hinge domain of the HPV11 E2 protein (33) and absent from the HPV18 and HPV16 E2 proteins. In addition, we showed that HPV18 E2 contains an NES in its amino-terminal domain that is responsible for active export of the protein from the nucleus to the cytoplasm (that was repressed by leptomycin B). Furthermore, inactivating point mutations of the strong

NLS of HPV11 E2 protein lead to a clear delocalization of the protein to the cytoplasm of transfected cells, indicating that this NLS is sufficient to explain the strict nuclear localization of the full-length proteins. In contrast, more complex phenomena probably dictate the localization of the HPV18 E2 as none of the NLSs appeared sufficient to induce nuclear retention. In contrast, a NES is able to delocalize the protein to the cytoplasm, so the subcellular localization of the protein seems to be tightly regulated by a complex interplay of signals and active nucleo-cytoplasmic shuttling.

As transcription and replication factors, E2 proteins should be primarily expressed in the nuclei of infected cells during the normal viral life cycle. Other functions of the high risk virus E2 proteins could account for their cytoplasmic localization, and we have shown that their proapoptotic activity could be one of them. There are clear differences between the E2 proteins of the high risk or low risk viruses in inducing apoptosis. Although E2 proteins from both high risk viruses were able to induce apoptosis at equivalent rates in different cell types, the E2 proteins from low risk viruses were unable to do so. Caspase 8 activation could be detected in both HPV16- and HPV18 E2-expressing cells, indicating that the mechanism of apoptotic cell death was probably conserved between these two viruses. In addition, using mutants of the E2 proteins, we could show that apoptosis is linked to the cytoplasmic expression of E2, linking apoptosis to the nucleo-cytoplasmic shuttling rather than to inherent properties of the E2 protein.

There are several other viral proteins that have been shown to activate apoptosis of infected cells through various mechanisms (for review, see Refs. 38–40), although the exact role of this specific function in the viral life cycle is generally not fully understood. Among these viral proteins, there are some that have been shown to induce apoptosis through caspase 8 activation, such as the lyssavirus matrix protein (41), the NS3 protein of hepatitis C (42), or HBx of hepatitis B. HBx, among various pleiotropic functions, is a transcription factor that is expressed both in the nucleus and in the cytoplasm of infected cells. The cytoplasmic HBx protein can activate caspase 8 by repressing c-FLIP through direct protein interaction, rendering the cells hypersensitive to tumor necrosis-α apoptotic activation (43). Nucleo-cytoplasmic shuttling of proteins is a frequent event accompanying induction of apoptosis. Many apoptotic proteins have to be expressed in a specific cellular compartment to be efficient and probably to allow direct protein-protein interactions. Examples of viral proapototic proteins, which are expressed both in the cytoplasm and in the nucleus of infected cells, are apoptin, the viral protein associated with chicken anemia and E4orf4 of adenovirus. Contrary to E2, apoptin has to be expressed in the nucleus to exert its proapoptotic function (44). In contrast, E4orf4 exerted its proapoptotic activity by two distinct pathways, one in the nucleus and the other in the cytoplasm (45). Caspase 8 activation occurs via the extrinsic death pathway as a consequence of the activation of the death-inducing signal complex through death receptors at the cytoplasmic membrane (46). Because E2 is an intracellular protein, caspase activation might occur within the cytoplasm by direct action on the proteins of the death-inducing signal complex or directly on caspase 8 itself. We do not yet know the exact mechanism by which E2 activates apoptosis, but it is clearly due to direct interactions of E2 with cytoplasmic substructures containing activated caspase 8 (results not shown).

Why might viruses induce apoptosis of the infected cells? A plethora of viral proteins inducing apoptosis has been described indicating that the proapoptotic functions play a universal role during the viral life cycle (for review, see Refs. 38–40 and 47). One possibility is that apoptosis might help the newly created particles at the end of the viral life cycle to escape from the host cell and spread out. This hypothesis, however, raises the question of why this function is not conserved in low risk HPVs. There are at least two possibilities. First, the low risk E2 proteins could be more efficient in inducing apoptosis in a more physiological situation, in co-expression with other viral proteins, for instance, that could help E2 to shuttle to the cytoplasm. This is a particularly attractive hypothesis, since we have shown that the low risk HPV E2 proteins can induce apoptosis if they are artificially directed to the cytoplasm of infected cells. Another possibility is that this function is expressed by another viral protein, such as E4 for instance, which is a viral protein expressed at later time points in the viral cycle and which has been shown to induce apoptosis (48). An alternative hypothesis is that E6 and E7 proteins of high risk HPVs modify the cell cycle and block cellular differentiation. These modifications of the host cell biology might induce a greater resistance to apoptosis, specifically with the reduction of p53 expression (49). The proapoptotic activity of the high risk E2 proteins would be required to overcome these effects, contrary to the low risk infected cells, where many of the E6 and E7 functions are not conserved. The proapoptotic activity of high risk E2 proteins, however, could explain why the high risk viral genomes integrated into the cellular DNA preferentially with disruption of the E2 ORF, with this disruption leading to higher transcription of the E6 and E7 oncogenes and carcinogenic progression.

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