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RAPID COMMUNICATION

Comparative Analysis of the Intracellular Location of the High- and Low-Risk Human Papillomavirus Oncoproteins

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We have compared the intracellular location of the HPV E6 and E7 proteins from high- and low-risk virus types. While high-risk HPV E7 displays diffuse nuclear expression, low-risk E7 has a nuclear punctuate pattern of expression. Similarly, while high-risk E6 is expressed throughout the cell, low-risk E6 is again predominantly nuclear with a punctuate pattern of expression. Both low-risk viral oncoproteins show colocalization with PML, whereas high-risk viral proteins do not. Finally, inhibition of the proteasome pathway results in a dramatic nuclear accumulation of high-risk E6 protein. These results demonstrate fundamental differences in the localization of these viral oncoproteins within the cell and offer alternative explanations for their respective differences in pathology.

Key Words: HPV; E6; E7; proteasome.

Introduction. Human papillomaviruses (HPVs) are intimately associated with the development of cervical cancer (*26*). Over 100 different HPV types have now been identified, but only a small subset of these are associated with the development of human cancer (*5*). Based on this, the HPV types are classified as either high or low risk, depending on whether they are associated with malignant or benign lesions, respectively (*26*). In cervical cancer the predominant viral types found are HPV-16 and HPV-18, whereas in benign lesions of the cervix HPV-6 and HPV-11 are most frequently found. High-risk HPVs encode two oncoproteins, E6 and E7, both of which have oncogenic activity in a variety of cell transformation assays (*2, 11*). Perhaps the most important of these activities is the ability to bring about the immortalization of primary cervical keratinocytes (*1*), which requires the presence of both E6 and E7. A causal role for these two proteins in the development of cervical cancer is also supported by the fact that both viral proteins continue to be retained and expressed in the cervical tumors and derived cell lines many years after the initial immortalization events (*17*). Indeed, numerous studies have now been performed to show that continued expression of these two viral proteins is essential for maintenance of the transformed phenotype (*3, 24*).

Indications as to the mechanism of action of these two viral proteins have come from many studies that have investigated their interactions with cellular target proteins. Thus, E7 interacts with a number of cell cycle regulatory proteins, in particular, the pRb family of pocket proteins (*7*). In the case of E6, the first cellular target identified was the cellular tumor suppressor p53 (*25*). Over the past few years many other cellular targets of E6 and E7 have been identified (see *13, 15*, for reviews). Based on the complex pattern of interactions, taken together with the biological activities of these two viral proteins, a consensus scenario is emerging in which E7 contributes principally to the induction of DNA synthesis and cell cycle progression, whereas E6 contributes to increased cell survival and progression to malignancy (*20*).

A major aim of many of these studies has been to attempt to define the underlying reasons that E6 and E7 from the high-risk virus types are transforming, whereas E6 and E7 from the low-risk virus types are not. Some of the differences are clearly linked to their respective abilities to interact with their cellular targets. However, there is very little information on whether the high- and low-risk viral oncoproteins actually localize to the same cellular compartments. Obviously, differences at such a basic level of function could profoundly influence their abilities to contribute to cell transformation. Therefore we have performed a series of studies to compare the cellular localization of the E6 protein of HPV-11 with that of HPV-18 and the E7 protein from HPV-11 with that of HPV-16.

Results. Localization of the HPV-11 and HPV-16 E7 proteins. Previous studies had reported a predominantly nuclear localization for the HPV-16 E7 protein (*19*), but

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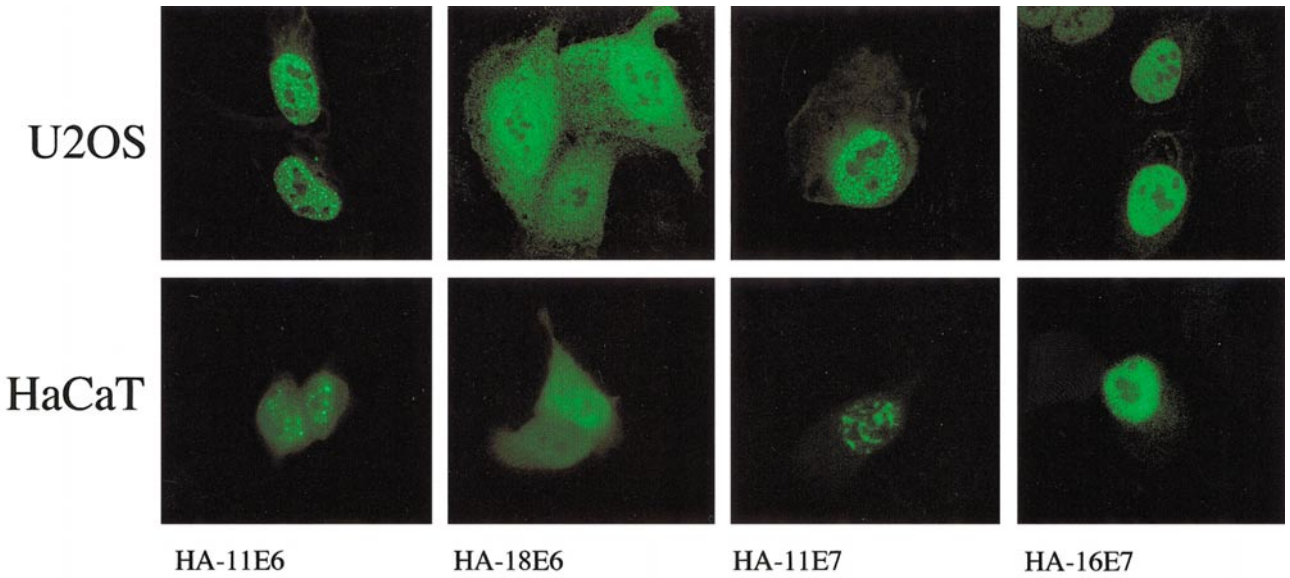
there are no reports of the cellular localization of the HPV-11 E7 protein. To investigate the comparative pattern of expression of the two E7 proteins, U2OS and HaCaT cells were transfected with plasmids expressing HA-tagged HPV-16 E7 and HA-tagged HPV-11 E7. After 24 h cells were fixed and stained with an anti-HA polyclonal antibody, and patterns of E7 expression were assessed using confocal microscopy. The results obtained are shown in Fig. 1. In agreement with previous studies, the HPV-16 E7 protein exhibits a largely nuclear pattern of expression with nucleolar exclusion. Similarly, HPV-11 E7 is also largely confined to the nucleus and, most importantly, the pattern of expression of both proteins is similar regardless of the cell type. It is also clear, however, that the low-risk E7 protein has regions of concentration within the nucleus, with the presence of clear dot-like structures. Very similar patterns of expression have been observed for proteins that localize the PML oncogenic domains (PODs) (*21*). Therefore we proceeded to investigate whether the two E7 proteins were indeed localizing to PODs. Cells were again transfected with the HPV-11 and HPV-16 E7 expression plasmids and after 24 h the cells were fixed and stained for E7. The results obtained are shown in Fig. 2. Again HPV-11 E7 shows nuclear localization, with a distinct punctuate pattern of expression. It is also clear from the costaining with anti-PML antibody that a significant proportion of the HPV-11 E7 protein colocalizes with PODs (see bottom left-hand corner of the nucleus). In contrast, it is clear that although the HPV-16 E7 protein is predominantly nuclear, it does not colocalize with PML. These results demonstrate a significant difference in the precise patterns of expression of the HPV-11 and HPV-16 E7 proteins within the nucleus; HPV-11 E7 localizes to POD structures, whereas the HPV-16 E7 protein does not.

Localization of the HPV-11 and HPV-18 E6 Proteins. There are conflicting reports on the cellular location of the high-risk HPV E6 proteins, with expression being detected in different cellular locations (*4, 12, 18*). In contrast there have been no studies on the pattern of expression of the low-risk E6 proteins. Therefore we proceeded to compare the pattern of expression of HPV-11 E6 and HPV-18 E6. HaCaT and U2OS cells were transfected with plasmids containing HA-tagged versions of the two proteins and cells were fixed and stained after 24 h. The results obtained are shown in Fig. 1 and show a striking difference in the patterns of expression in both cell types. As can be seen, HPV-11 E6 is largely confined to the nucleus and, like HPV-11 E7, exhibits a marked punctuate pattern of expression. In contrast, HPV-18 E6 is much more diffusely expressed throughout the cell, and this is consistent with previous reports demonstrating its presence within a variety of different cellular compartments (*4, 12, 18*).

Since we found that the HPV-11 E7 protein localized to POD structures and that HPV-11 E6 also displays a somewhat similar punctuate pattern of expression, we proceeded to investigate whether HPV-11 E6 was also localizing to PODs. Cells were again transfected with HA-tagged HPV-11 and HPV-18 E6 and after 24 h the cells were fixed and stained with anti-HA and anti-PML antibodies. The results obtained are shown in Fig. 2. As can be seen, a significant proportion of HPV-11 E6 also shows colocalization with PML (see left-hand side of the nucleus), demonstrating that it also localizes to POD structures. In contrast, in the case of HPV-18 E6 it is clear that the protein does not colocalize with PODs. Therefore these results demonstrate major differences in the precise patterns of cellular localization of the high- and low-risk associated viral oncoproteins.

HPV-18 E6 Localization is an Actively Controlled Process. The HPV-18 E6 protein has been shown to be intimately involved in a number of pathways linked to proteasome-mediated degradation of its cellular substrate proteins (*9, 16, 23*). Therefore we decided to investigate whether the pattern of HPV-18 E6 expression might be affected by proteasome inhibition. Cells were transfected with HA-tagged HPV-11 and HPV-18 E6 proteins, and after 24 h, the cells were treated with the specific proteasome inhibitor CBZ (MG132), for 90 min. The cells were then fixed and stained with anti-HA antibody. The results obtained are shown in Fig. 3. In each set DMSO was used as negative control showing no differences with respect to untreated cells (data not shown). It is clear that there is minimal change in either the pattern or the intensity of HPV-11 E6 protein expression. The protein remains predominantly nuclear and retains the clear punctuate pattern of expression confocal with PML and with the POD-associated protein SUMO-1. However, in the case of HPV-18 E6, there is a striking relocalization of the protein after proteasome inhibition. Following treatment of the cells with MG132, HPV-18 E6 is almost entirely relocated to the nucleus. In addition, it is striking that the sites of nuclear accumulation of HPV-18 E6 following CBZ treatment are still largely not confocal with either PML or SUMO-1. This nuclear accumulation of HPV-18 E6 occurs very rapidly and, as can be seen from the time-course in Fig. 4, can be detected after 10 min of proteasome inhibition. By 90 min of proteasome inhibition the majority of the HPV-18 E6 protein is present within the nucleus. These results demonstrate that the localization of HPV-18 E6 within the cell is regulated by dynamic cellular processes, which, when blocked by proteasome inhibition, result in nuclear accumulation of the HPV-18 E6 protein.

Discussion. In this study we have compared the intracellular location of the HPV E6 and E7 oncoproteins from the low- and the high-risk viral types and show fundamental differences in the precise cellular localization of these viral proteins. The low-risk HPV-11 E6 and E7



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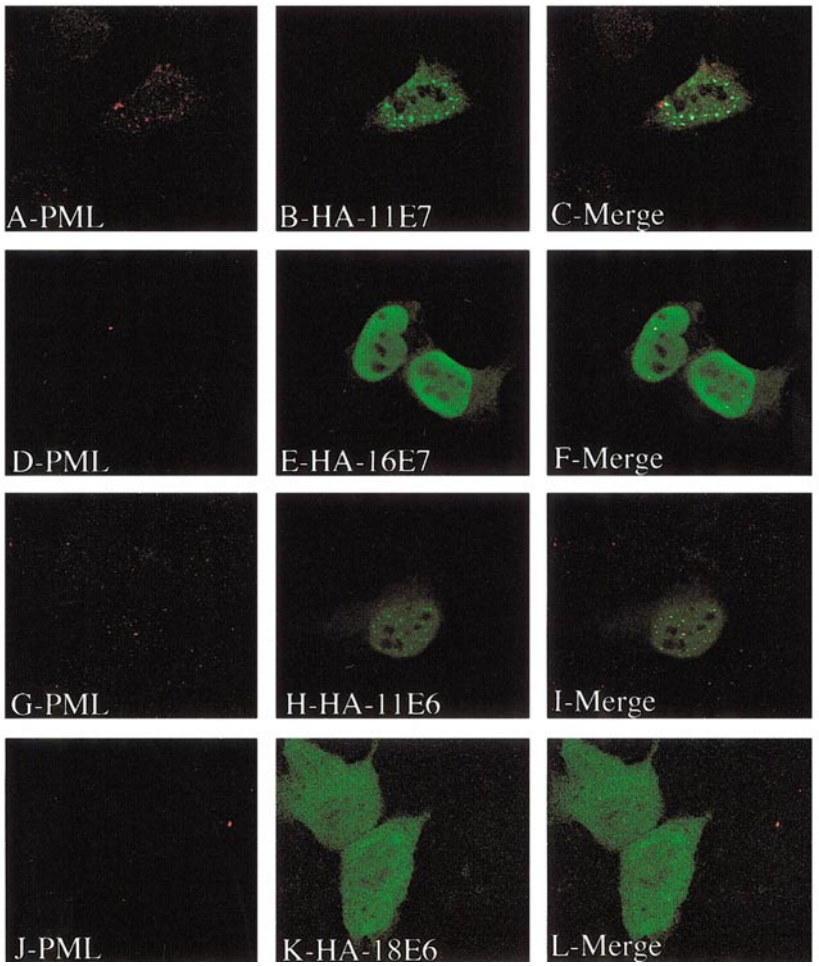

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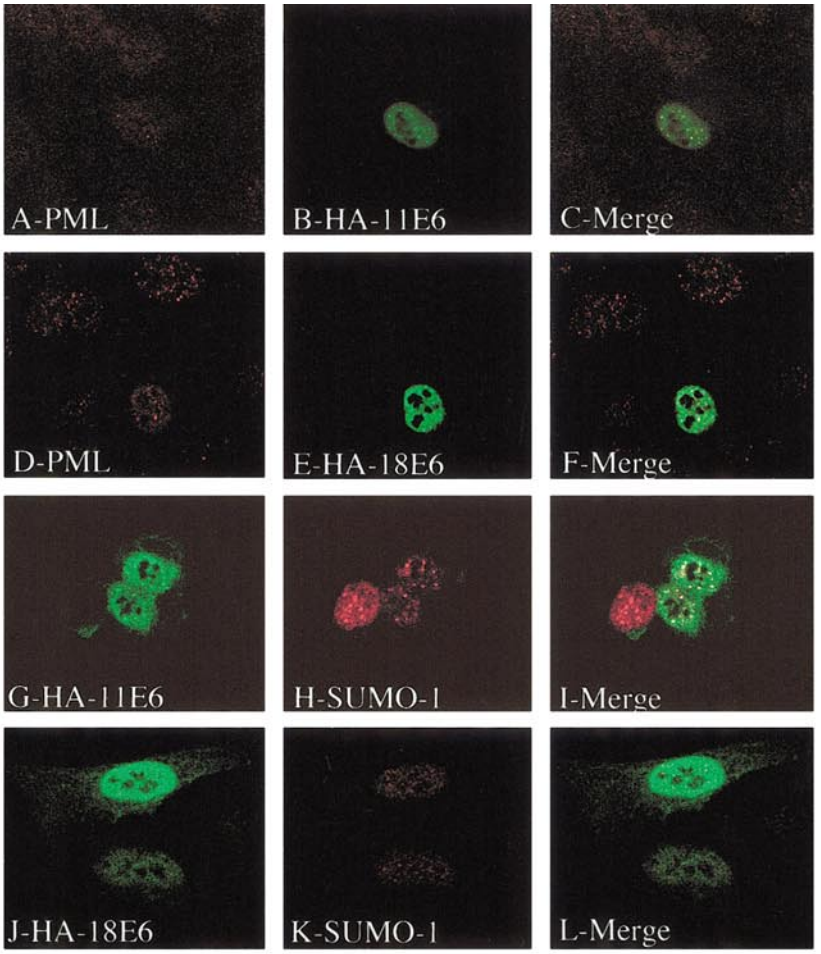
FIG. 1. HA-E7 and HA-E6 localization in transiently transfected U2OS and HaCaT cells. The cells are fixed in 3% PFA, permeabilized with Triton X-100, and stained for HA-11E6, HA-18E6, HA-11E7, and HA-16E7 as indicated.

FIG. 2. HA-E7 and HA-E6 localization in transiently transfected U2OS cells. U2OS cells are fixed in 3% PFA, permeabilized with Triton X-100, and double-stained for HA-11E7 and PML (A–C) and HA-16E7 and PML (D–F) or for HA-11E6 and PML (G–I) and HA-18E6 and PML (J–L).



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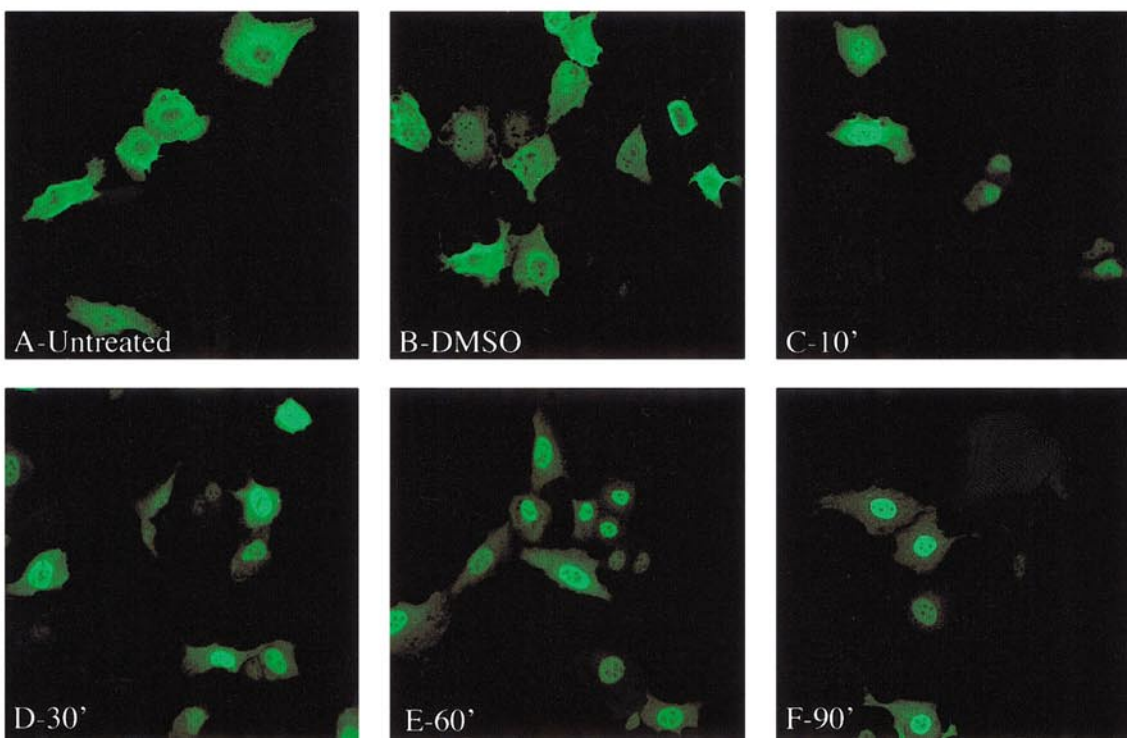

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FIG. 3. HA-E6 localization in transiently transfected U2OS cells following 90 min of CBZ treatment. U2OS cells are fixed in 3% PFA, permeabilized with Triton X-100, and double-stained for HA-11E6 and PML (A–C) and HA-18E6 and PML (D–F) or for HA-11E6 and SUMO-1 (G–I) and HA-18E6 and SUMO-1 (J–L).

FIG. 4. Time course of HA-18E6 nuclear relocalization. U2OS cells were transiently transfected with HA-18E6 and treated for an increasing amount of time (10–90 min) with CBZ (C–F). Untreated cells and cells treated for 90 min with DMSO are also shown as controls (A, B).

proteins have a tendency to accumulate in dot-like structures within the nucleus and we have demonstrated that these correspond to PODs. In contrast, the high-risk HPV-16 E7 protein, while also localizing predominantly to the nucleus, does not accumulate in PODs. Most interestingly, however, the high-risk HPV-18 E6 protein has a very different pattern of cellular localization, being found throughout the cell. These studies therefore provide alternative explanations for the profound differences in the pathology of the two virus types.

An important caveat to these studies is the issue of epitope tagging and functionality of the proteins. Although it has not been possible to address this for the low-risk HPV proteins, we know that the epitope-tagged HPV-18 E6 retains the ability to degrade MAGI-1 (*10*) and p53 (data not shown) *in vivo,* demonstrating that the tag does not adversely affect E6 function and localization. We have also attempted to address the issue of potential artifacts from overexpression first by ensuring that, where comparisons were made, equivalent levels of expression were observed, and second by performing the comparisons between different cells exhibiting high and low levels of fluorescence. In all cases there are consistent differences in the pattern of expression of the high- and low-risk viral oncoproteins. It will now be interesting to extend this analysis to other HPV types, but preliminary studies with HPV-16 E6 show an identical pattern of expression to that obtained with HPV-18 E6 (data not shown).

The finding that the low-risk E6 and E7 proteins both accumulate in POD structures but that the high-risk proteins do not is intriguing. The precise role of these cellular structures is still unclear, but numerous studies have shown that targeting viral proteins to these domains is an important step in viral replication (*8, 14*). In addition, previous studies have found that sites of DNA replication of the low-risk HPV types are also concentrated close to PODs (*22*). This suggests that the low-risk virus types may have evolved an efficient mechanism to make use of cellular POD structures, whether it be for viral packaging or for utilizing the numerous cellular proteins which accumulate there. In contrast, the high-risk HPV proteins do not appear to accumulate anywhere near as efficiently within these POD structures. Therefore this difference in the pattern of cellular localization may provide a partial explanation as to why the high-risk virus replication cycle is somewhat delayed in comparison with that of the low-risk viruses (*6*).

The most striking difference however, lies in the completely different patterns of localization seen with the low- and high-risk E6 proteins. HPV-11 E6 is mainly nuclear, while the HPV-18 E6 protein is distributed in many compartments of the cell, with a degree of membrane localization. This latter point is particularly important, since numerous studies have shown that the high-risk E6 proteins can target several cellular proteins which have a membrane localization (*4, 9, 23*) and this has been something of a contentious issue for some time. It would also appear that the function of the high-risk E6 proteins has evolved quite differently from that of the low-risk proteins. Thus, high-risk E6 proteins are distributed throughout the cell, thereby allowing interactions to take place with a more diverse group of cellular proteins. It therefore seems likely that this is a fundamental reason that the high-risk E6 proteins induce many more phenotypic changes within the cell compared with their low-risk counterparts.

Finally, it is also clear that the localization of HPV-18 E6 within the cell is a highly controlled process. Inhibition of the proteasome degradation pathway results in a dramatic relocalization of the HPV-18 E6 protein to the nucleus, which occurs over a very short time, being clearly visible within 10 min after addition of inhibitor. This suggests that it may represent active nuclear import of already expressed E6, rather than a failure of nuclear export. Studies are now in progress to determine the mechanism behind this nuclear accumulation of HPV-18 E6.

Materials and Methods. Cells and transfections. U2OS and HaCaT cells were grown in DMEM plus 10% heat-inactivated fetal calf serum. Transfections were done either by calcium phosphate precipitation or by Superfect (Qiagen). For the proteasome inhibition experiments cells were grown for different amounts of time in the presence of 50 μM carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (CBZ/MG132) diluted in DMSO (Sigma) or DMSO alone as a negative control.

Plasmids. HA-tagged HPV-11 and HPV-18 E6 expression vectors were kindly provided by Ron Javier. HA-tagged HPV-16 E7 and HPV-11 E7 were cloned by PCR amplification and the products were verified by DNA sequencing.

Immunofluorescence. Twenty-four hours after transfection cells were fixed in 3% paraformaldehyde in PBS for 20 min at room temperature, washed once with 0.1 M glycine, permeabilized with 0.1% Triton X-100 in PBS, and then incubated with the primary antibody diluted 1:200 for 1 h. PML was detected with a mouse monoclonal antibody (Santa Cruz Biotechnology, Inc.). HA-tagged proteins were detected either with a polyclonal rabbit antibody (Santa Cruz Biotechnology, Inc.) in the cases of single staining and double staining with PML or with a mouse monoclonal antibody (Sigma) when double-stained with SUMO-1. SUMO-1 was detected using a polyclonal rabbit antibody (Santa Cruz Biotechnology, Inc.). The cells were then incubated with a 1:800 dilution of fluorescein-conjugated goat anti-rabbit antibody (Molecular Probes) and rhodamine red-conjugated goat anti-mouse antibody (Molecular Probes) for 20 min.

Confocal Microscopy. Slides were analyzed using a Zeiss LSM 510 confocal microscope with two lasers giving excitation lines at 543 and 488 nm. The data were collected at 1024 × 1024 pixel resolution. The microscope was a Zeiss Axiovert 100M with a 100× objective oil-immersion lens. Scanning conditions were kept constant in each experiment and ensured that the signal overlap between channels was essentially eliminated.

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REFERENCES

1. Barbosa, M. S., and Schlegel, R. (1989). The E6 and E7 genes of HPV-18 are sufficient for inducing two stage *in vitro* transformation of human keratinocytes. *Oncogene* **4,** 1529–1532.
2. Bedell, M., Jones, K., Grossman, S., and Laimins, L. (1989). Identification of human papillomavirus type 18 transforming genes in immortalised and primary cells. *J. Virol.* **63,** 1247–1255.
3. Butz, K., Denk, C., Ullmann, A., Scheffner, M., and Hoppe-Seyler, F. (2000). Induction of apoptosis in human papillomavirus-positive cancer cells by peptide aptamers targeting the viral E6 oncoprotein. *Proc. Natl. Acad. Sci. USA* **97,** 6693–6697.
4. Chen, J., Reid, C., Band, V., and Androphy, E. (1995). Interaction of papillomavirus E6 oncoproteins with a putative calcium binding protein. *Science* **269,** 529–531.
5. De Villiers, E. M. (1994). Human pathogenic papillomavirus types: An update. *Curr. Top. Microbiol. Immunol.* **186,** 1–12.
6. Doorbar, J., Foo, C., Coleman, N., Medcalf, L., Hartley, O., Prospero, T., Napthine, S., Sterling, J., Winter, G., and Griffin, H. (1997). Characterization of events during the late stages of HPV16 infection *in vivo* using high-affinity synthetic Fabs to E4. *Virology* **238,** 40–52.
7. Dyson, N., Guida, P., Münger, K., and Harlow, E. (1992). Homologous sequences in adenovirus E1A and human papillomavirus E7 proteins mediate interaction with the same set of cellular proteins. *J. Virol.* **66,** 6893–6902.
8. Everett, R. D., and Maul, G. G. (1994). HSV-1 IE protein Vmw 110 causes redistribution of PML. *EMBO J.* **13,** 5062–5069.
9. Gardiol, D., Kühne, C., Glaunsinger, B., Lee, S. S., Javier, R., and Banks, L. (1999). Oncogenic human papillomavirus E6 proteins target the discs large tumour suppressor for proteasome-mediated degradation. *Oncogene* **18,** 5487–5496.
10. Glaunsinger, B. A., Lee, S. S., Thomas, M., Banks, L., and Javier, R. (2000). Interaction of the PDZ-protein MAGI-1 with adenovirus E4-ORF1 and high-risk papillomavirus E6 oncoproteins. *Oncogene* **19,** 5270–5280.
11. Kanda, T., Furuno, A., and Yoshiike, K. (1988). Human papillomavirus type 16 open reading frame E7 encodes a transforming gene for rat 3Y1 cells. *J. Virol.* **62,** 610–613.
12. Kanda, T., Watanabe, S., Zanma, S., Sato, H., Furuno, A., and Yoshiike, K. (1991). Human papillomavirus type 16 E6 proteins with glycine substituted for cysteine in metal binding motifs. *Virology* **185,** 536–543.
13. Mantovani, F., and Banks, L. (2001). The human papillomavirus E6 protein and its contribution to malignant progression. *Oncogene* **20,** 7874–7887.
14. Muller, S., and Dejean, A. (1999). Viral immediate-early proteins abrogate the modification by SUMO-1 of PML and Sp100 proteins, correlating with nuclear body disruption. *J. Virol.* **73,** 5137–5143.
15. Münger, K., Basile, J. R., Duensing, S., Eichten, A., Gonzalez, S. L., Grace, M., and Zacny, V. L. (2001). Biological activities and molecular targets of the human papillomavirus E7 oncoprotein. *Oncogene* **20,** 7888–7898.
16. Scheffner, M., Werness, B., Huibregtse, J., Levine, A., and Howley, P. (1990). The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* **63,** 1129–1136.
17. Schwarz, E., Freese, U., Gissmann, L., Mayer, W., Roggenbuck, B., Stremlau, A., and zur Hausen, H. (1985). Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. *Nature* **314,** 111–114.
18. Sherman, L., and Schlegel, R. (1996). Serum- and calcium-induced differentiation of human keratinocytes is inhibited by the E6 oncoprotein of human papillomavirus type 16. *J. Virol.* **70,** 3269–3279.
19. Smith-McCune, K., Kalman, D., Robbins, C., Shivakumar, S., Yuschenkoff, L., and Bishop, J. M. (1999). Intranuclear localization of human papillomavirus 16 E7 during transformation and preferential binding of E7 to the Rb family member p130. *Proc. Natl. Acad. Sci. USA* **96,** 6999–7004.
20. Song, S., Liem, A., Miller, J. A., and Lambert, P. F. (2000). Human papillomavirus type 16 E6 and E7 contribute differently to carcinogenesis. *Virology* **267,** 141–150.
21. Sternsdorf, T., Grotzinger, T., Jensen, K., and Will, H. (1997). Nuclear dots: Actors on many stages. *Immunobiology* **198,** 307–331.
22. Swindle, C. S., Zou, N., Van Tine, B. A., Shaw, C. A., Engler, G. A., and Chow, L. T. (1999). Human papillomavirus DNA replication compartments in a transient DNA replication system. *J. Virol.* **73,** 1001–1009.
23. Thomas, M., and Banks, L. (1998). Inhibition of Bak-induced apoptosis by HPV-18 E6. *Oncogene* **17,** 2943–2954.
24. von Knebel Doeberitz, M., Rittmuller, C., zur Hausen, H., and Dürst, M. (1992). Inhibition of tumorigenicity of cervical cancer cells in nude mice by HPV E6–E7 anti-sense RNA. *Int. J. Cancer* **51,** 831–834.
25. Werness, B., Levine, A., and Howley, P. (1990). Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science* **248,** 76–79.
26. zur Hausen, H. (1991). Human papillomaviruses in the pathogenesis of anogenital cancer. *Virology* **184,** 9–13.