Involvement of Nuclear Export in Human Papillomavirus Type 18
E6-Mediated Ubiquitination and Degradation of p53

Deborah Stewart,1,2 Anirban Ghosh,1 and Greg Matlashewski1*
Department of Microbiology and Immunology, McGill University, Montreal, Canada,1 and Institute of Parasitology, McGill University, Montreal, Canada2

Received 1 November 2004/Accepted 15 March 2005

The E6 protein from high-risk human papillomaviruses (HPVs) targets the p53 tumor suppressor for degradation by the proteasome pathway. This ability contributes to the oncogenic potential of these viruses. However, several aspects concerning the mechanism of E6-mediated p53 degradation at the cellular level remain to be clarified. This study therefore examined the role of cell localization and ubiquitination in the E6-mediated degradation of p53. As demonstrated within, following coexpression both p53 and high-risk HPV type 18 (HPV-18) E6 (18E6) shuttle from the nucleus to the cytoplasm. Mutation of the C-terminal nuclear export signal (NES) of p53 or treatment with leptomycin B inhibited the 18E6-mediated nuclear export of p53. Impairment of nuclear export resulted in only a partial reduction in 18E6-mediated degradation, suggesting that both nuclear and cytoplasmic proteasomes can target p53 for degradation. This was also consistent with the observation that 18E6 mediated the accumulation of polyubiquitinated p53 in the nucleus. In comparison, a p53 isoform that localizes predominantly to the cytoplasm was not targeted for degradation by 18E6 in vivo but could be degraded in vitro, arguing that nuclear p53 is the target for E6-mediated degradation. This study supports a model in which (i) E6 mediates the accumulation of polyubiquitinated p53 in the nucleus, (ii) E6 is coexported with p53 from the nucleus to the cytoplasm via a CRM1 nuclear export mechanism involving the C-terminal NES of p53, and (iii) E6-mediated p53 degradation can be mediated by both nuclear and cytoplasmic proteasomes.

The p53 protein is a tumor suppressor that can cause cell cycle arrest or apoptosis in response to DNA damage and other forms of stress (19). The critical role played by p53 in regulating normal cell growth is highlighted by the observations that p53-deficient mice are rendered highly susceptible to sporadic cancers (6) and germ line mutations in p53 result in Li-Fraumeni syndrome, in which individuals are predisposed to a variety of cancer types (22). Furthermore, approximately 50% of tumors contain mutated p53 genes (15), while the remaining 50% frequently appear to have compromised p53 function. For example, wild-type p53-containing tumors may overexpress the mdm2 cellular oncogene or the E6 oncogene from high-risk human papillomavirus (HPV) types. Tight regulation of p53 activity involves a variety of posttranslational modifications including ubiquitination, proteasome-mediated degradation, and control of subcellular localization. Mdm2, a cellular ubiquitin ligase (16), is an important negative regulator of p53 activity and mediates the ubiquitin-dependent degradation of p53 by the proteasome (13, 17).

High-risk HPV types, such as types 16 and 18, are the etiological agents for the development of cervical cancer (34). The oncogenic potential of these viruses correlates with the in vitro transforming activities of the viral oncoproteins E6 and E7. The E7 protein promotes the upregulation of proliferation-related genes (25) through its interaction with the retinoblastoma gene product, pRb, and related proteins (5, 7). E6, alternatively, circumvents the cell’s apoptotic response to uncontrolled cell proliferation by binding to p53 (31) and targeting the protein for degradation (27).

The ability to localize to the nucleus is essential for p53 to act as a transcription factor. Modification of the subcellular distribution of p53 therefore represents an important regulatory mechanism. Numerous studies have provided strong evidence that nuclear export of p53 to the cytoplasm is required for its efficient degradation by Mdm2 (2, 8, 10, 26). However, other studies suggest that Mdm2 targets p53 for degradation by nuclear proteasomes (32, 33). A more recent study argues that the nuclear-cytoplasmic shuttling of p53 is required for Mdm2-mediated cytoplasmic degradation, but not ubiquitination (24). The Mdm2-mediated translocation of ubiquitinated p53 to the cytoplasm may in fact act as a rapid, initial regulatory step in inhibiting p53 transcriptional transactivation.

While considerable attention has focused on defining the role of nuclear-cytoplasmic shuttling in the Mdm2-mediated ubiquitination and degradation of p53, few studies have explored this notion in the context of the HPV E6-p53 interaction. An early study observed stabilization of p53 in HPV-negative and -positive cancer-derived cell lines in the presence of the CRM1 nuclear export inhibitor leptomycin B (LMB) (8). These results argued that nuclear export was required for both Mdm2- and high-risk HPV-16 E6-mediated degradation of p53. Recently, the ability of HPV E6 to preferentially localize to the nucleus in cervical cancer cells suggests that it may rely on nuclear-cytoplasmic shuttling events in order to efficiently inhibit the activity of a number of transcriptional regulator targets, including p53 (23). However, it is unclear what role nuclear-cytoplasmic shuttling plays in the E6-mediated degradation of p53, and in which cellular compartment E6-mediated ubiquitination and degradation of p53 occur. This
study therefore sought to further clarify the role of cell localization and ubiquitination in the E6-mediated inactivation of p53.

**MATERIALS AND METHODS**

**Cell lines and transfections.** Murine p53-null 10(1) fibroblast cells derived from the NIH 3T3 cell line and human p53-null H1299 cells, kindly provided by Sam Benchimol, were used in this study. Cells were maintained in Dulbecco's modified Eagle's medium (GIBCO) with 10% fetal bovine serum (GIBCO) and 100 units/ml penicillin-streptomycin (GIBCO). Lipofectamine (GIBCO) was used to transfect cells according to the manufacturer's protocol. A luc expression plasmid was included in all transfections, and β-galactosidase activity was determined in transfected cells to control for variation in transfection efficiencies. For LMB (Sigma) experiments, either 20 ng/ml of LMB or solvent (70% ethanol) was added to the cell media 16 h posttransfection, and cell lysates were prepared 24 h posttransfection.

**Construction of p53 and E6 expression plasmids.** The green fluorescence protein (GFP)-E6 fusion proteins, containing GFP at the N-termini, were generated as previously described (29). The p53-GFP expression construct was kindly provided by Geoffrey Workman (30) and expresses a p53-GFP fusion protein containing GFP at the C-terminal end of the protein. The C-terminal nuclear export signal (NES) mutant of p53 [p53(351-493)] was generously provided by Carl Maki (10). This mutant contains leucine-to-alanine conversions at positions 348 and 350. Plasmids coexpressing p53 and N-terminally deleted p53 (p57) [called p53(EII)], and p57 alone, have recently been described in detail (11).

**Detection of p53 and GFP fusion proteins.** Proteins were expressed in vitro using the transcription/translation system (Promega) according to the manufacturer's instructions. To determine the effect of HPV E6 expression on the localization of the p53-GFP fusion protein, cell localization was partially as previously described (18). Briefly, H1299 cells were cotransfected with p53 or p53(30)-expressing vectors and either control pCDNA3, HPV E6, or Mdm2 expression vectors and a hemagglutinin (HA)-tagged ubiquitin expression vector. The proteasome inhibitor MG-132 (20 μM) (Calbiochem) was added 4 h before harvesting the cells, and cell lysates were prepared on ice 24 h posttransfection by lysis in buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, protease inhibitor cocktail [Roche]). N-Ethylmaleimide (NEM) (Sigma) was included in the lysis buffer and all subsequent immunoprecipitation and wash buffers at a final concentration of 5 mM to inactivate components of the ubiquitin system (including those involved in ubiquitination [17a] and deubiquitination [9]). Cell debris was discarded after centrifugation at 14,000 rpm for 10 min at 4°C. Lysates were precleared with normal mouse serum and incubated at 4°C for 2 h with anti-p53 mAb DO-1 (1:10,000) followed by a 1:10 volume of protein A-Sepharose for 30 min. Immunoprecipitates were washed four times with cold lysis buffer. Loading volumes for the washed immunoprecipitates were standardized for equal transfection efficiencies according to β-galactosidase activity, and samples were analyzed by Western blot using mouse monoclonal anti-HA conjugated antibody (Roche).

**Preparation of nuclear fractions.** Nuclear enriched fractions were prepared as previously described (20), with minor changes. Briefly, cells were harvested in 5 volumes of cold HB buffer (10 mM Tris, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, protease inhibitor cocktail, 5 mM NEM) and incubated on ice for 15 min, followed by the addition of 0.2% final concentration of Triton X-100. Cells were vortexed for 5 s and centrifuged at 14,000 rpm for 3 min at 4°C. The supernatant, representing the cytoplasmic fraction, was transferred to a fresh tube, and the salt concentration was adjusted to 250 mM. The crude pellet was washed four times in cold HB buffer and was resuspended in buffer C (10 mM Tris, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 400 mM NaCl, 1% Triton X-100, protease inhibitor cocktail, 5 mM NEM) and incubated on ice for 25 min. Following vigorous vortexing for 5 min at 4°C, the homogenate was centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant, representing the nuclear fraction, was transferred to a new tube, and the salt concentration was reduced to 200 mM with addition of an equal volume of HB buffer.

**Analysis of the nuclear levels of ubiquitinated p53 and p53(NES-) involved the same transfection procedure as described above. Preclearing and immunoprecipitation of the nuclear fractions using anti-p53 mAb DO-1 antibody were also performed as described above. Enriched nuclear fractions were analyzed by anti-topoisomerase I antibodies (Santa Cruz Biotechnologies Inc.) to confirm enrichment of the nuclear fraction.**

**RESULTS**

**HPV-18 E6 alters the cellular localization of p53.** Initially, we determined whether HPV-18 E6 expression alters the cellular localization of p53 in live cells using a p53-GFP fusion protein. Prior to this analysis however, it was necessary to confirm that high-risk HPV-18 E6 is able to efficiently mediate the degradation of the p53-GFP fusion protein. Furthermore, it was essential to confirm that no detectable GFP is released following degradation, which could lead to a misinterpretation of the localization results. 10(1) cells, null for p53, were cotransfected with a p53-GFP expression vector and either control plasmid or HPV-11 E6 or HPV-18 E6 expression vectors. Cell lysates were prepared 24 h posttransfection and p53 levels were determined by anti-GFP Western blot analysis. As shown in Fig. 1A, HPV-18 E6 but not HPV-11 E6 efficiently mediated the degradation of p53-GFP, and no free GFP was detected. To determine the effect of HPV E6 expression on the localization of the p53-GFP fusion protein, cell localization was determined 24 h posttransfection as previously described (2, 10, 11, 24). As demonstrated in Fig. 1B, high-risk HPV-18 E6 expression caused a redistribution of p53-GFP to a more cytoplasmic localization. In contrast, the low-risk HPV-11 E6 described (4). Equal amounts of p53 and p47 expression plasmid DNA were also included in the same TNT reaction for in vitro coexpression. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by anti-p53 Western blot analysis using mAb 1801.

**Ubiquitination assays.** The p53 ubiquitination assays were performed essentially as previously described (18). Briefly, H1299 cells were cotransfected with p53 or p53(NES-) expressing vectors and either control pCDNA3, HPV E6, or Mdm2 expression vectors and a hemagglutinin (HA)-tagged ubiquitin expression vector. The proteasome inhibitor MG-132 (20 μM) (Calbiochem) was added 4 h before harvesting the cells, and cell lysates were prepared on ice 24 h posttransfection by lysis in buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, protease inhibitor cocktail [Roche]). N-Ethylmaleimide (NEM) (Sigma) was included in the lysis buffer and all subsequent immunoprecipitation and wash buffers at a final concentration of 5 mM to inactivate components of the ubiquitin system (including those involved in ubiquitination [17a] and deubiquitination [9]). Cell debris was discarded after centrifugation at 14,000 rpm for 10 min at 4°C. Lysates were precleared with normal mouse serum and incubated at 4°C for 2 h with anti-p53 mAb DO-1 (1:10,000 dilution) followed by a 1:10 volume of protein A-Sepharose for 30 min. Immunoprecipitates were washed four times with cold lysis buffer. Loading volumes for the washed immunoprecipitates were standardized for equal transfection efficiencies according to β-galactosidase activity, and samples were analyzed by Western blot using mouse monoclonal anti-HA conjugated antibody (Roche).

**Preparation of nuclear fractions.** Nuclear enriched fractions were prepared as previously described (20), with minor changes. Briefly, cells were harvested in 5 volumes of cold HB buffer (10 mM Tris, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, protease inhibitor cocktail, 5 mM NEM) and incubated on ice for 15 min, followed by the addition of 0.2% final concentration of Triton X-100. Cells were vortexed for 5 s and centrifuged at 14,000 rpm for 3 min at 4°C. The supernatant, representing the cytoplasmic fraction, was transferred to a fresh tube, and the salt concentration was adjusted to 200 mM. The crude pellet was washed four times in cold HB buffer and was resuspended in buffer C (10 mM Tris, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 400 mM NaCl, 1% Triton X-100, protease inhibitor cocktail, 5 mM NEM) and incubated on ice for 25 min. Following vigorous vortexing for 5 min at 4°C, the homogenate was centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant, representing the nuclear fraction, was transferred to a new tube, and the salt concentration was reduced to 200 mM with addition of an equal volume of HB buffer.

**Analysis of the nuclear levels of ubiquitinated p53 and p53(NES-) involved the same transfection procedure as described above. Preclearing and immunoprecipitation of the nuclear fractions using anti-p53 mAb DO-1 antibody were also performed as described above. Enriched nuclear fractions were analyzed by anti-topoisomerase I antibodies (Santa Cruz Biotechnologies Inc.) to confirm enrichment of the nuclear fraction.**

**RESULTS**

**HPV-18 E6 alters the cellular localization of p53.** Initially, we determined whether HPV-18 E6 expression alters the cellular localization of p53 in live cells using a p53-GFP fusion protein. Prior to this analysis however, it was necessary to confirm that high-risk HPV-18 E6 is able to efficiently mediate the degradation of the p53-GFP fusion protein. Furthermore, it was essential to confirm that no detectable GFP is released following degradation, which could lead to a misinterpretation of the localization results. 10(1) cells, null for p53, were cotransfected with a p53-GFP expression vector and either control plasmid or HPV-11 E6 or HPV-18 E6 expression vectors. Cell lysates were prepared 24 h posttransfection and p53 levels were determined by anti-GFP Western blot analysis. As shown in Fig. 1A, HPV-18 E6 but not HPV-11 E6 efficiently mediated the degradation of p53-GFP, and no free GFP was detected. To determine the effect of HPV E6 expression on the localization of the p53-GFP fusion protein, cell localization was determined 24 h posttransfection as previously described (2, 10, 11, 24). As demonstrated in Fig. 1B, high-risk HPV-18 E6 expression caused a redistribution of p53-GFP to a more cytoplasmic localization. In contrast, the low-risk HPV-11 E6
FIG. 1. Relocalization of p53 and HPV-18 E6 to the cytoplasm when both proteins are coexpressed. (A) HPV-18 E6 efficiently targets p53-GFP for degradation, without the release of detectable free GFP. HPV-11 or -18 E6 expression vectors or the pCDNA3 control plasmid was cotransfected with a p53-GFP expression vector into p53-null 10(1) cells, as indicated, and subjected to Western blot analysis using an anti-GFP antibody. Note the presence of a faint band representing p53-GFP in cells coexpressing 18E6, and the absence of free GFP (expected molecular mass of approximately 25 kDa) in all lanes. Molecular weights (left) are in thousands. (B) Localization of p53-GFP in the presence of HPV E6. 10(1) cells were cotransfected with either control pCDNA3 or HPV-11 or -18 E6 expression vectors and the p53-GFP expression vector, as indicated. Cell localization was scored 24 h posttransfection. Dark grey bars represent the percentage of cells showing only nuclear fluorescence, light grey bars show the percentage of cells with a strong nuclear fluorescence and weak cytoplasmic fluorescence, and black bars represent the percentage of cells demonstrating equal or greater fluorescence in the cytoplasm. A minimum of 300 cells were scored per experiment, and results represent the means ± standard deviations from five independent experiments. (C) Representative photographs showing the localization of p53-GFP in either control or HPV-18 E6-expressing cells. (D) Localization of GFP-11E6 and GFP-18E6 in the presence of p53. 10(1) cells were cotransfected with GFP-11E6 or GFP-18E6 expression vectors, and either control pCDNA3 or a p53 expression plasmid, as indicated. Cell localization was scored 24 h posttransfection, as described above. (E) Representative photographs showing the localization of GFP-18E6 in the absence or presence of p53.
proteins had no effect on the predominantly nuclear localization of p53-GFP. Representative photographs for the localization of p53-GFP in the absence and presence of HPV-18 E6 (18E6) are shown in Fig. 1C. The reduced level of fluorescence of p53-GFP in cells coexpressing 18E6 (Fig. 1C) is consistent with the degradation observed in Fig. 1A, where only a faint band representing p53-GFP is detected.

To better define the mechanism of 18E6 relocalization of p53 to the cytoplasm, it was necessary to determine the effect of p53 on the localization of 18E6. HPV-11 and -18 E6 proteins were fused to GFP in order to detect E6 and perform the localization studies on live cells. We have previously confirmed that the GFP-18E6 fusion protein is similar to wild-type 18E6 with respect to targeting both p53 and hDLG for degradation (29). As shown in Fig. 1D, unlike GFP-11E6, which was unable to accumulate in the nucleus, high-risk GFP-18E6 was able to localize predominantly to the nucleus in approximately 35% of transfected cells. Coexpression of p53 reduced the percentage of cells showing a predominantly nuclear accumulation of GFP-18E6 from 35% to 16%. Cotransfection of p53 had no effect on the localization of GFP-11E6. Representative photographs showing the cellular distribution of GFP-18E6 in the absence and presence of p53 are contained in Fig. 1E. Taken together, the observations contained in Fig. 1 show that 18E6 mediated a redistribution of p53 from the nucleus to the cytoplasm and that this was accompanied by an increase in the cytoplasmic localization of 18E6.

Nuclear export is required for HPV-18 E6 to relocate p53-GFP to the cytoplasm. It was necessary to determine whether the redistribution of p53 from the nucleus to the cytoplasm in the presence of 18E6 was due to an increase in nuclear export or a reduction in nuclear import. We therefore investigated whether impairment of CRM1-associated nuclear export inhibited the cytoplasmic accumulation p53-GFP and whether this was associated with a suppression of 18E6-mediated p53 degradation. 10(1) cells were cotransfected with p53-GFP and HPV E6 expression vectors. Cells were treated with the CRM1 nuclear export inhibitor LMB for 8 h prior to determination of the cellular localization and level of p53-GFP at 24 h posttransfection. As shown in Fig. 2A, LMB treatment significantly increased the nuclear localization of p53-GFP in the absence of E6. Consistent with results contained in Fig. 1, 18E6 was able to relocalize p53-GFP to a more cytoplasmic distribution in the absence of LMB. In contrast, the ability of 18E6 to relocalize p53-GFP from the nucleus to the cytoplasm was inhibited by LMB treatment. Western blot analysis with anti-GFP antibody revealed that in the presence of LMB 18E6
Mediated Nuclear Export of p53.

Taken together, these data demonstrate that 18E6-mediated p53 degradation occurred at a reduced level under conditions where 18E6 was unable to efficiently relocate p53-GFP from the nucleus to the cytoplasm. These results are consistent with a previous report in showing that nuclear export is required for the efficient degradation of p53 by high-risk HPV E6 (8). However, since a significant level of 18E6-mediated p53 degradation occurred in the presence of LMB, the data in Fig. 2 also suggest that 18E6 was able to mediate p53 degradation in the nucleus. To further explore this possibility, we next investigated whether the C-terminal NES of p53 was required for 18E6-mediated nuclear export of p53 and whether 18E6 retained the ability to mediate the degradation of a p53(NES-) mutant.

The C-terminal NES of p53 is required for HPV-18 E6-mediated nuclear export of p53. It has been previously established that the C-terminal NES of p53, but not the NES of Mdm2, is essential for Mdm2-mediated nuclear export of p53 (2, 10). Based on the localization and degradation results obtained using LMB, as presented above, it was necessary to establish the importance of the C-terminal NES of p53 for E6-mediated nuclear export and degradation. Human H1299 cells, null for p53, were cotransfected with either wild-type p53 or p53 containing a mutation in its C-terminal NES [p53(NES-)] (10), and the effect of HPV-18 E6 on the localization of both wild-type p53 and p53(NES-) was determined. The effect of Mdm2 on the localization of wild-type and mutant p53 was included as a control to confirm whether this experimental system yielded results consistent with those previously reported (10). As demonstrated in Fig. 3A, similar to results obtained with p53-GFP in murine 10(1) cells (Fig. 1B and 2A), 18E6 was able to efficiently relocate wild-type p53 from the nucleus to the cytoplasm. Mdm2 was also able to induce wild-type p53 nuclear export, as expected. However, in the presence of either 18E6 or Mdm2, there was a significant inhibition of p53(NES-) nuclear export when compared to wild-type p53. Representative photographs showing the cellular localization of p53(NES-) in the absence and presence of 18E6 are contained in Fig. 3B.

Consistent with the observation in Fig. 1D, coexpression of p53 increased the percentage of cells with cytoplasmically localized GFP-18E6 (Fig. 3C). In contrast, coexpression of the p53(NES-) mutant did not alter GFP-18E6 localization, which remained identical to control cells. The data contained in Fig. 3A to C demonstrate that the C-terminal NES of p53 is required for 18E6-mediated p53 nuclear export.

To determine whether 18E6 could mediate degradation of the p53(NES-) mutant, Western blot analysis of p53 was performed. As shown in Fig. 3D, 18E6 was able to efficiently mediate degradation of the p53(NES-) mutant, although at a reduced level compared to wild-type p53. Note that in this analysis an addition control was included, in the form of the 18E6ΔM mutant (delta 28-31), which is unable to mediate p53 degradation. The ability of Mdm2 to partially target p53(NES-) for degradation is also consistent with the report by Geyer and colleagues (10).

Taken together, these results demonstrate that, while the C-terminal NES of p53 was required for 18E6-mediated nuclear export of p53 and p53-mediated nuclear export of 18E6, this element was not absolutely essential for 18E6-mediated degradation of p53. These results are therefore in agreement with those contained in Fig. 2 in arguing that 18E6 is able to mediate the degradation of p53 in both the nucleus and cytoplasm.

HPV-18 E6 is unable to target cytoplasmically sequestered p53 for degradation. Several studies have identified the expression of a truncated isoform of p53, termed p47, which lacks the first N-terminal 43 amino acids and can arise by alternative splicing of the p53 gene (11), or by alternative initiation of translation on the same p53 transcript (3a, 32a). In contrast to p53, p47 localizes predominantly in the cytoplasm and mediates the nuclear export of p53 in an Mdm2-independent manner (11). Since the N-terminally truncated p53 (p47) retains the E6 binding- and degradation-required sites (21), it represents a useful p53 isoform to determine whether the colocalization of E6 and p53 in the nucleus is necessary for E6-mediated p53 degradation. H1299 cells were cotransfected with expression plasmids for HPV-11 E6, HPV-18 E6, and either p53 or p47 expression plasmids. Cell lysates were analyzed by anti-p53 Western blot using a monoclonal antibody (mAb 1801), which recognizes both p53 and the truncated p47 isoform. As demonstrated in Fig. 4A, 18E6 was unable to target p47 for degradation under cellular conditions where it effectively mediated p53 degradation. Note that low-risk 11E6 was unable to mediate the degradation of either p53 or p47.

As shown previously, coexpression of p47 and p53 mediates the cytoplasmic accumulation of p53 (11), and it was therefore of interest to further investigate whether 18E6 could target cytoplasmically sequestered wild-type p53 for degradation. Western blot analysis was performed on cell lysates from H1299 cells cotransfected with the various E6 expression plasmids, together with a plasmid construct expressing equal amounts of both p53 and p47. As shown in Fig. 4B, even when threefold more E6 to p53/p47 expression plasmid was transfected, 18E6 was unable to target p53 (or p47) for degradation when p53 was expressed in the presence of p47.

Based on the above observations, it was necessary to confirm the cell localization of p47 in the presence and absence of 18E6. Likewise, it was necessary to determine the cell localization of p53 when it was coexpressed with p47 in the presence and absence of 18E6. As shown in Fig. 4C, p47 was predominantly localized to the cytoplasm and coexpression of 18E6 had little effect on the cellular distribution of p47. Likewise, p53 in the presence of p47 was predominantly localized to the cytoplasm both in the absence and presence of 18E6. In comparison, p53 expressed in the absence of p47 was predominantly present in the nucleus and coexpression with 18E6 resulted in a shift from the nucleus to the cytoplasm, consistent with results presented above.

We also determined whether p47, or p53 in the presence of p47, could shift the cell localization of GFP-18E6 from the nucleus to the cytoplasm. As shown in Fig. 4D, neither p47, nor p53 in the presence of p47, altered the localization of GFP-18E6 under conditions where p53 alone could shift GFP-18E6 to a more cytoplasmic localization.

An explanation for the above observations is that the cytoplasmic sequestration of p47, or p53 in the presence of p47, inhibits their interaction with 18E6 in the nucleus, resulting in
the inability of 18E6 to target either the wild-type or N-terminally truncated p53 (p47) for degradation. In order to investigate this hypothesis, it was necessary to determine whether 18E6 could mediate the in vitro degradation of p47, or p53 in the presence of p47, under conditions where the proteins were expressed using an acellular, in vitro transcription/translation system. In an in vitro degradation assay, the proteins are homogenously distributed and are able to interact in the absence of potential compartmentalization effects present in a cellular environment. As demonstrated in Fig. 5A, 18E6 was able to target both p53 and p47 for degradation in vitro when they were expressed individually. The ability of 18E6 to target p53...
for degradation more efficiently than p47 in vitro may be associated with minor changes in wild-type conformation due to the deletion of the amino terminus in the p47 protein. Importantly, when p53 and p47 proteins were first coexpressed in vitro, 18E6 effectively targeted both proteins for degradation (Fig. 5B). As expected, 11E6 was unable to target p53 or p47 for degradation in vitro. These in vitro degradation results are in sharp contrast to those observed in vivo in transfected cells, in which 18E6 was unable to target either p47, or p53 in the presence of p47, for degradation. Taken together, the data presented in Fig. 4 and 5 support the argument that, in a cellular environment, cytoplasmic p47, and cytoplasmic p53 in the presence of p47, were unable to complex with 18E6 in the nucleus and therefore 18E6 was unable to mediate their degradation. However, in the acellular, in vitro degradation assays, p47 and p53 were able to associate with 18E6 and this resulted in the 18E6-mediated degradation of wild-type and N-terminally truncated p53.

HPV-18 E6-mediated ubiquitination of p53 in the nucleus. While the nuclear export of p53 by Mdm2 has been shown to be dependent upon the ubiquitination of p53 (10, 21a), the relationship between HPV E6-mediated in vivo ubiquitination and the nuclear export of p53 has not been established. As demonstrated in Fig. 3A, the C-terminal NES of p53 was required for 18E6-mediated nuclear export of p53. We therefore determined if the lack of nuclear export of p53(NES-) was due to reduced E6-mediated ubiquitination. Following cotransfections with a plasmid expressing HA-tagged ubiquitin, whole-cell lysates were immunoprecipitated with anti-p53 mAb DO-1 antibody, followed by Western blot analysis using anti-HA antibody to detect the HA-tagged ubiquitin-conjugated p53. For these analyses, transfected cells were incubated in the

FIG. 4. Western blot analysis of HPV-18 E6-mediated degradation of cytoplasmically sequestered p53 and p47. (A) H1299 cells were cotransfected with the indicated plasmids expressing p53 (left panel) or p47 (right panel) together with pCDNA3 plasmid (control) or increasing amounts of either HPV-11 or -18 E6 expression plasmids (at 1:1 or 1:3 ratios). Cell lysates were prepared 24 h posttransfection and were subjected to Western blot analysis using anti-p53 mAb 1801. (B) The experiment was performed as described for panel A except that the p53(EII) plasmid expressing both p53 and p47 proteins was cotransfected into H1299 cells, with either control pCDNA3 or E6 expression plasmids, as indicated. (C) Immunofluorescence (IF) analysis was performed using mAb DO-1, specific for p53, or mAb 1801 to detect p47 in cells expressing only p47, as detailed in Materials and Methods. H1299 cells were cotransfected with p53, p53(EII) (expressing both p53 and p47) or p47 expression plasmids and either the control pCDNA3 or HPV-18 E6 expression plasmid, as indicated. Cell localization was determined 24 h posttransfection. Dark grey bars represent the percentage of cells showing only nuclear fluorescence, light grey bars show the percentage of cells with a strong nuclear fluorescence and weak cytoplasmic fluorescence, and black bars represent the percentage of cells demonstrating equal or greater fluorescence in the cytoplasm. Note that p47 or p53 in the presence of p47 (p53/p47) localize predominantly to the cytoplasm, and 18E6 does not influence the localization of p47 or p53 in the presence of p47. (D) Cell localization of GFP-18E6 in the presence of p53, p47, or p53/p47. H1299 cells were cotransfected with a GFP-18E6 expression plasmid and either p53, p53(EII) (expressing both p53 and p47), or p47 expression plasmids, as indicated. Cell localization was determined 24 h posttransfection. Note that expression of p47 or coexpression of p53 and p47 (p53/p47) inhibited p53-mediated nuclear export of GFP-18E6.
The presence of the proteasome inhibitor MG-132 for 4 h prior to harvesting, to facilitate detection of 18E6-mediated ubiquitin-conjugated p53. As shown in Fig. 6A, 18E6 mediated similar levels of ubiquitination of both p53 and p53(NES-) (lanes 3 and 7). These data show that the inability of 18E6 to mediate the nuclear export of p53(NES-) was not due to a reduction in the ubiquitination of p53(NES-) relative to wild-type p53. Under these conditions, Mdm2 mediated higher overall levels of ubiquitination of both p53 and p53(NES-), compared to 18E6 (compare lanes 3 and 4, and 7 and 8). Consistent with the inability of 11E6 to target p53 for degradation, 11E6 was also unable to mediate p53 ubiquitination (lane 2). Anti-p53 Western blot analysis of the whole-cell lysates used for immunoprecipitations is shown in Fig. 6B.

Detection of 18E6-mediated ubiquitination of p53(NES-) and the requirement of p53 to localize to the nucleus for susceptibility to 18E6-mediated degradation prompted us to verify whether 18E6 mediated the accumulation of ubiquitinated p53 and p53(NES-) in the nucleus. We were also interested to determine whether we could detect higher levels of 18E6-mediated p53 ubiquitination in a nuclear enriched fraction since, relative to Mdm2, only low levels of 18E6-mediated p53 ubiquitination were detectable in the total cell lysates. Following transfection and enrichment of the nuclear fraction, ubiquitination of p53 and p53(NES-) was analyzed as described above (Fig. 6C). Nuclear and cytoplasmic fractions (5% input) were subjected to anti-topoisomerase I Western blot analysis to confirm enrichment of the nuclear fraction (Fig. 6D). As shown in Fig. 6C, 18E6 mediated similar levels of ubiquitinated p53 and p53(NES-) in these nuclear preparations (lanes 2 and 5). Moreover, in contrast to the total cell lysates (Fig. 6A), coexpression of 18E6 or Mdm2 resulted in similar levels of p53 ubiquitination in the nuclear fractions (compare lanes 2 and 3 and 5 and 6). Similar results were observed in the absence of proteasome inhibitor; however the amount of ubiquitinated p53 detected was reduced (data not shown). These data show that 18E6 mediates an accumulation of ubiquitinated p53 in the nucleus. Interestingly, whereas Mdm2 mediated both mono- and polyubiquitination, 18E6 appeared to mediate predominantly polyubiquitination of p53. Taken together, the data from Fig. 6 confirm that 18E6 mediates the accumulation of polyubiquitinated p53 in the nucleus and that this is consistent with the data presented in Fig. 2 and 3, arguing that 18E6 can also mediate p53 degradation by nuclear proteasomes.

DISCUSSION

While Mdm2 is the main regulator of p53 levels in normal cells (16), this pathway is nonfunctional in HPV-positive cervical carcinoma cells, and p53 degradation is entirely dependent upon E6 function (13a). However, the mechanism of E6-mediated p53 degradation at the cellular level has not been clearly defined. The observations made in this study reveal several relevant and novel aspects of E6-mediated degradation, including (i) high-risk HPV-18 E6 but not low-risk HPV-11 E6 is able to accumulate predominantly in the nucleus, and both 18E6 and p53 shuttle from the nucleus to the cytoplasm when coexpressed; (ii) the p53 C-terminal NES is required for 18E6-mediated p53 degradation at the cellular level; (iii) impairment of nuclear export with LMB or mutation of the p53 C-terminal NES results in a reduction, but not complete inhibition, of 18E6-mediated p53 degradation, arguing that 18E6 can target p53 for degradation by both nuclear and cytoplasmic proteasomes; (iv) impairment of the nuclear localization of p53 inhibits 18E6-mediated p53 degradation, arguing that both 18E6 and p53 must colocalize in the nucleus prior to 18E6-mediated degradation; and (v) 18E6 mediates the accumulation of polyubiquitinated p53 in the nucleus. Taken together, these observations support a model in which assembly of the...
FIG. 6. HPV-18 E6-mediated ubiquitination of p53 and p53(NES-). (A) H1299 cells were cotransfected with wild-type p53 or p53(NES-) expression plasmids, an HA-tagged ubiquitin expression plasmid, and either control pCDNA3 plasmid or HPV-11 E6, HPV-18 E6, or Mdm2 expression plasmids, as indicated. To facilitate detection of ubiquitin-conjugated p53, 20 μM MG-132 was added to cells at 20 h posttransfection, and lysates were prepared 24 h posttransfection. Lysates were immunoprecipitated with anti-p53 mAb DO-1, and Western blot analysis was performed using anti-HA antibody to detect ubiquitin-conjugated p53 proteins, as detailed in Materials and Methods. Approximate regions of monoubiquitinated (mono-Ub) and polyubiquitinated (poly-Ub) p53 are indicated, based on those previously reported (20). Molecular weights (left) are in thousands. (B) Western blot analysis using mAb 1801 was performed to determine p53 protein levels in the whole-cell lysates (5% input), which were used to carry out the ubiquitination analysis shown in panel A. Note that MG-132 treatment reduced 18E6- and Mdm2-mediated degradation of p53 and p53(NES-) (lanes 3 and 4 and 7 and 8), as expected. (C) HPV-18 E6-mediated ubiquitination of p53 and p53(NES-) in the enriched nuclear fraction. H1299 cells were cotransfected with wild-type p53 or p53(NES-) expression plasmids, an HA-tagged ubiquitin expression plasmid, and either control pCDNA3 plasmid, HPV-18 E6 or Mdm2 expression plasmids, as indicated. Nuclear fractions were prepared 24 h posttransfection and p53 was immunoprecipitated with anti-p53 mAb DO-1. Western blot analysis was performed using anti-HA antibody to detect ubiquitin-conjugated p53, as detailed in Materials and Methods. Molecular weights (left) are in thousands. (D) Nuclear (N) and remaining cytoplasmic (C) fractions (5% input) for each of the immunoprecipitated nuclear fractions were analyzed by Western blot using anti-topoisomerase I antibody to confirm nuclear enrichment.
E6/E6AP/p53 complex occurs in the nucleus and p53 is poly-
ubiquitinated and is exported to the cytoplasm with E6 via a process
dependent upon the C-terminal NES of p53 and the CRM1 nuclear export
pathway. Degradation of p53 can, however, occur in both the nucleus and cytoplasm.

The present study significantly extends our understanding of E6-mediated p53 degradation at the cellular level. Previous studies argued that E6 requires nuclear export in order to target p53 for degradation, based on the inhibition of E6-mediated p53 degradation in cervical cancer-derived cells treated with the CRM1 nuclear export inhibitor, LMB (8, 14). The present study identifies that the p53 C-terminal NES is necessary for E6-mediated nuclear export, and mutation of this signal had a similar effect as LMB treatment in impairing nuclear export. This was an important observation to confirm the relationship between E6-mediated nuclear export and degradation of p53, in light of a previous study which reported that at least part of the LMB-mediated stabilization of p53 in cer-
vical cancer-derived cells was likely due to a reduction in E6-E7 mRNA levels, and not an inhibition of nuclear export (14). In addition, HPV-18 E6-mediated ubiquitination and degradation of p53 appear to be independent events, as approximately equal levels of E6-mediated ubiquitination of wild-type and mutant p53(NESS-)-proteins were observed, despite the reduced ability of E6 to target p53(NESS-) for export and degradation.

It was also revealed in this study that the C-terminal NES of p53 is required for the nuclear export of 18E6 when co-
expressed with p53. This is in contrast to the Mdm2-dependent p53 degradation pathway, in which Mdm2 remains exclusively nuclear and does not shuttle to the cytoplasm with p53 (10). However, similar to 18E6, Mdm2 also requires the p53 C-
terminal NES to mediate p53 nuclear export (10). As demonstrated herein, Mdm2 and 18E6 also mediate different major
forms of ubiquitinated p53 in the nucleus. While Mdm2 mediates both p53 mono- and polyubiquitination, 18E6 predomi-
nantly mediates the polyubiquitination of p53, which was readily detectable in the nuclear enriched fraction. Interest-
ingly, a previous study was unable to detect E6-mediated p53 ubiquitination in vivo (3) suggesting the possibility that the cellular degradation of p53 by E6 may not involve ubiquitina-
tion. Camus and colleagues (3), however, did not examine E6-mediated p53 ubiquitination in the nuclear fraction. As shown within, 18E6-mediated p53 ubiquitination was much easier to detect in the nuclear fraction than in the total cell
lysate.

It has recently been shown that monoubiquitinated p53 is

targeted for nuclear export but not degradation, while poly-
ubiquitinated p53 is targeted for degradation (20), and we have
made similar observations (11). Following Mdm2-mediated monoubiquitination of p53, polyubiquitination may then occur in the cytoplasm, possibly facilitated by an E4 ligase such as p300 (12), which ultimately leads to p53 degradation. Consis-
tent with this model, Mdm2 is able to induce the nuclear export of p53 while remaining in the nucleus (10) and appears to be able to target p53 for degradation by both nuclear and cyto-
plasmic proteasomes (28, 32, 33). These observations suggest that 18E6 and Mdm2 mediate p53 export by different mecha-

nisms, where Mdm2 achieves export through mediating p53 monoubiquitination and 18E6 mediates the export of poly-
ubiquitinated p53 through a mechanism that requires the co-export of 18E6. However, inhibition of nuclear export by mu-

tation of the C-terminal NES of p53 or LMB treatment did not
completely inhibit E6-mediated p53 degradation, arguing that the degradation of polyubiquitinated p53 can occur by both nuclear and cytoplasmic proteasomes.

This study demonstrates that nuclear export is not absolutely necessary for E6-mediated p53 degradation to occur. However, the ability of p53 to accumulate in the nucleus is required for degradation since the cytoplasmic sequestration of p53, mediated by its N-terminally truncated isoform (p47), provided virtually complete protection against 18E6-mediated degrada-
tion. Likewise, 18E6 was unable to mediate the degradation of p47, which also localizes predominantly to the cytoplasm. It is important to note that p47 retains the E6-binding and degra-
dation-dependent sequences (21), which is consistent with the results presented in this study that 18E6 could mediate the degradation of p47 in vitro. Taken together, these observations argue that the association between E6 and p53 must occur in the nucleus before E6-mediated degradation of p53 can pro-
ceed.

The ability of E6 to target p53 for ubiquitination in the nucleus, accompanied by moderate levels of degradation, sug-
gests that therapeutic strategies that aim to simply sequester p53 in the nucleus of HPV-positive cells may not be an effectiv-
e strategy. Approaches attempting to either inhibit E6 ex-
pression or prevent p53-E6 association may therefore repre-
sent better therapeutic alternatives.

ACKNOWLEDGMENTS

We are grateful to C. Maki for kindly providing us with the
p53(NESS)-expression plasmid, G. Wahl for the p53-GFP expression plasmid, D. Bohmann for the HA-ubiquitin expression plasmid, and S. Wing for the kind gift of N-ethylmaleimide. We also thank J. Nadeau for assistance with the epifluorescent microscopy.

This work was supported by the National Sciences and Engineering Research Council of Canada (NSERC). G.M. holds a Canadian Instit-
utes of Health Research Senior Investigator Award, and D.S. has been supported by NSERC and FCAR student fellowships.

REFERENCES

p53-specific monoclonal antibodies and their use in the studies of human p53


3. Camus, S., M. Higgins, D. Lane, and S. Lain. 2003. Differences in the
224.

Oren, and P. Hainaut. 2002. ΔN-p53, a natural isoform of p53 lacking the
first transactivation domain, counters growth suppression by wild-type p53.

of transcriptional regulatory properties of p53 by HPV E6. Oncogene
9:1225–1230.

papillomavirus type 16 E7 associates with a histone H1 kinase and with p107


ломavirus-16 E7 oncoprotein is able to bind to the retinoblastoma gene


2001. c-myc overexpression activates alternative pathways for intracellular