The Human Papillomavirus 16 E6 Protein Binds to Tumor Necrosis Factor (TNF) R1 and Protects Cells from TNF-induced Apoptosis

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High risk strains of human papillomavirus (HPV), such as HPV 16, cause human cervical carcinoma. The E6 protein of HPV 16 mediates the rapid degradation of p53, although this is not the only function of E6 and cannot completely explain its transforming potential. Previous work in our laboratory has demonstrated that transfection of HPV 16 E6 into the tumor necrosis factor (TNF)-sensitive LM cell line protects expressing cells from TNF-induced apoptosis in a p53-independent manner, and the purpose of this study was to determine the molecular mechanism underlying this protection. Caspase 3 and caspase 8 activation were significantly reduced in E6-expressing cells, indicating that E6 acts early in the TNF apoptotic pathway. In fact, E6 binds directly to TNF R1, as shown both by co-immunoprecipitation and mammalian two-hybrid approaches. E6 requires the same C-terminal portion of TNF R1 for binding as does TNF R1-associated death domain, and TNF R1/TNF R1-associated death domain interactions are decreased in the presence of E6. HA-E6 also blocked cell death triggered by transfection of the death domain of TNF R1. Together, these results provide strong support for a model in which HPV E6 binding to TNF R1 interferes with formation of the death-inducing signaling complex and thus with transduction of proapoptotic signals. They also demonstrate that HPV, like several other viruses, has developed a method for evading the TNF-mediated host immune response.

High risk strains of human papillomavirus (HPV), such as HPV 16, cause most cases of human cervical carcinoma (reviewed in Ref. 1). HPV 16 codes for two oncoproteins, E6 and E7. The E7 protein functions by binding to and inactivating the tumor suppressor protein Rb, while E6 is best known for mediating the rapid degradation of the tumor suppressor p53. Whereas this activity clearly contributes to the oncogenic potential of E6, this viral protein has additional biological and transforming activities that appear to be independent of p53 (2–10). Mechanisms of E6 action probably involve interaction with cellular proteins, and indeed, the HPV E6 protein has been reported to interact with a number of cellular proteins in addition to p53 and E6-AP (reviewed in Ref. 11). These include proteins involved in the regulation of transcription and DNA replication, such as p300/CREB-binding protein (12, 13), IRF-3 (14), hMcm7 (15, 16), and E6TP1 (17); proteins involved in apoptosis such as Bak (18) and c-Myc (19); proteins involved with epithelial organization and differentiation such as paxillin (20) and E6BP/ERC-55 (21); and proteins involved in cell-cell adhesion, polarity, and proliferation control that contain a PDZ binding motif (X(T/S)XV) such as hDLAG (22, 23), hScrib (24), MAGI-1 (25, 26), and MUPPI (27). However, for most of these binding partners, the effect of the E6 interactions on the virus life cycle or its capacity to transform host cells is not well understood.

Another area not yet well understood is the molecular mechanism(s) underlying the lack of a vigorous immune response to papillomavirus infections. Papillomaviruses are persistent viruses that remain in their hosts for long periods of time and elicit a weak or undetectable specific immune response and little or no inflammatory response. It may well be that one or more of the interactions between virus and cellular proteins contribute to this evasion of the host immune response. Tumor necrosis factor (TNF) is capable of inducing apoptosis of cells infected by some viruses (reviewed in Ref. 28). Current understanding of molecular mechanisms responsible for TNF-mediated apoptosis begins with the binding of the trimeric TNF molecule to the 55-kDa TNF receptor 1 (TNF R1). This initiates interactions between TNF R1-associated death domain (TRADD) and Fas-associated death domain (FADD), which in turn interact with procaspase 8 (FLICE) to activate the caspase cascade, ultimately resulting in apoptosis (for reviews, see Refs. 23–25). The complex responsible for initiating this process is known as the death-inducing signaling complex (DISC). TNF does not induce apoptosis of every cell to which it binds; in fact, most cells are protected (29). The reason for this is that TNF also triggers a separate, protective pathway involving activation of NF-κB transcription factor family members, which counteracts the cytolytic actions of TNF in many cells (30, 31). The effect of TNF on a given cell, therefore, is determined by the activity in both the proapoptotic and the prosurvival pathways.
In previous work, we found that transfection of HPV 16 E6 into TNF-sensitive mouse fibroblast LM cells resulted in a blockade of TNF-induced apoptosis by a p53-independent mechanism (32). In this study, we tested the hypothesis that E6 mediates this apoptosis blockade by interacting with one or more proteins in pathways triggered by TNF signaling. We demonstrated that E6 interacts with TNF R1 and interferes with its capacity to bind TRADD and to transmit a proapoptotic signal. These findings provide a mechanistic explanation for the capacity of E6 to protect cells from TNF-triggered cell death and also suggest that papillomaviruses have developed a way to evade this arm of the host immune system.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Lyophilized human recombinant TNF-α (R & D Systems, Minneapolis, MN), was dissolved into serum-free minimal essential medium to yield a 1 μg/ml stock, aliquoted, and stored at −80 °C until use. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was dissolved in phosphate-buffered saline to yield a 5 mg/ml stock and stored at 4 °C until use. Mitomycin C (Sigma) was dissolved in Me2SO to yield a 5 mg/ml stock and stored at 4 °C until use. Cycloheximide (Sigma) was prepared as a 5 mg/ml stock and stored in aliquots at −20 °C prior to use. C2-ceramide (P.B. Cargo, PA) was dissolved in Me2SO to yield a 25 mM stock solution and stored at −80 °C prior to use. Anti-HA was obtained from Roche Molecular Biochemicals.

**Cell Culture**—LM (mouse fibroblast), U2OS (human osteosarcoma), U937 (human histiocyte/monocyte), and NIH3T3 (mouse fibroblast) cells were obtained from the ATCC (Manassas, VA). LME6 cells were derived by co-transfection of LM cells with pSV2neo an pSG16E6, which encodes the E6 gene from HPV 16 (32). LM and NIH3T3 cells were cultured in minimal essential medium (Invitrogen). LME6 cells were cultured in minimal essential medium containing G418 (800 μg/ml) (Invitrogen). U937 cells were cultured in RPMI 1640, and U2OS cells were cultured in McCoy’s 5A medium (Invitrogen). All culture medium was supplemented to contain 10% fetal bovine serum (Invitrogen).

**Plasmids**—pSV2neo contains a gene for G418 resistance (33). pSG5 contains an expression cassette for neomycin resistance (34). pCS5 is a eukaryotic constitutive expression vector (Stratagene, La Jolla, CA) that includes the early region SV40 promoter for in vivo expression and the T7 promoter for in vitro transcription. pSG16E6 (a gift from Lamo- ninis Laaimins, Northwestern University Medical School, Chicago, IL) is derived from pSG5 and incorporates the wild-type sequence from the E6 gene of HPV type 16.

A plasmid expressing epitope-tagged HPV 16 E6 was generated by first inserting the cytomegalovirus promoter (from pC1 neo, Promega, Madison, WI) into the BglII-EcoRI site of the promoterless plasmid pEGFP-1 (CLONTECH, Palo Alto, CA). The PCR product of E6 (derived from plasmid pSG16E6) was then cloned into HA tag Bluescript KS in either the sense (pHA-E6 S) or the antisense orientation (pHA-E6 AS).

**Cell Death Assay**—The cell death detection ELISA (Roche Molecular Biochemicals) was used to measure mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates according to the manufacturer’s instructions. Cell lysates were added to wells to which the anti-histone antibody was fixed adsorptively. Nucleosomes in the sample bound via their histone components to the immobilized anti-histone antibody. Anti-DNA-enzyme, which binds to the DNA portion of the nucleosomes, was then added. The amount of bound peroxidase was determined photometrically by measuring absorbance at 405 nm after the addition of 2,2’-azino-di(3-ethylbenzthiazolin sulphonate) as substrate. Each point was measured in triplicate, and results were normalized to the untreated control. Because the different cell lines each displayed different levels of background cell death, this normalization provided a better comparison between the lines.

**Cell Viability Assay**—Two different versions of the MTT assay were used: one for adherent cells (LM and U2OS-derived cells) and one for suspension cells (U937-derived cells). For adherent cells, the incubation medium was removed and exchanged for 80 μl of fresh medium. 20 μl of MTI was then added (5 μg/ml stock), and cells were incubated at 37 °C for 24 h. Ceramide was then added to the indicated final concentration, and cells were incubated for 16 h prior to measurement of the number of viable cells by the MTT assay.

**Immunoblot Assays**—Cells (2 × 10^5) were lysed in 100 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 μM NaCl, 1% Triton X-100, 1 mM EDTA, 1 μM dithiothreitol, 100 μg/ml phenylmethylsulfonyl fluoride). One tablet of protease inhibitor mixture (Roche Molecular Biochemicals) per 10 ml of buffer was added just prior to use. The protein concentration in cleared lysates was measured using the BCA assay (described below). Lysates were then centrifuged (10,000 rpm for 10 min) to pellet the cells near the bottom of the wells. The top 240 μl of medium was removed, leaving 60 μl/well, and 15 μl of MTI (5 μg/ml stock) was added. The cells were incubated at 37 °C for 3 h. Two volumes (150 μl) of isopropanol/HCl (400 μl of HCl plus 100 ml of isopropanol) were added, and the cells were incubated for 10 min. The solution was mixed by pipetting, and the absorbance of each well was determined at 490 nm.

For suspension cells, plates were centrifuged (1000 rpm for 10 min) to pellet the cells near the bottom of the wells. The top 240 μl of medium was removed, leaving 60 μl/well, and 15 μl of MTI (5 μg/ml stock) was added. The cells were incubated at 37 °C for 3 h. Two volumes (150 μl) of isopropanol/HCl (400 μl of HCl plus 100 ml of isopropanol) were added, and the cells were incubated for 10 min. The solution was mixed by pipetting, and the absorbance of each well was determined at 490 nm.

**Transfections**—Transfections were carried out using Fugene VI (Roche Molecular Biochemicals), as directed by the manufacturer. For stable transfections, cells were seeded into 18-mm diameter dishes, transfected with 18 μg of DNA, and allowed to recover for 24 h. For stable transfections, clones were passaged into selection medium containing G418 (500 μg/ml) 72 h post-transfection. Individual clones were selected, grown, and analyzed for protein expression by immunoblotting.
A slurry was then washed three times with lysis buffer, followed by one wash with high salt buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, 1 mM dithiothreitol) and one wash with buffer lacking NaCl. The precipitates were fractionated by 12% SDS-PAGE, and immunoblotting was performed as described above.

Reverse Transcriptase-PCR—The reverse transcriptase polymerase chain reaction was used to analyze the U2OS and U2OS/SE617 cell lines for the presence of mRNA coding for E6. 3.5 μg of total RNA was isolated from each cell line and used as a template. cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) and an oligo (dT) primer (Amersham Biosciences). Primers for the 5’-GCACCAAAAGAGAACTGCAATGT-3’ and 5’-TGGTTTCTCTACGTGTCTTCTGAT-3’ were used to amplify the 468-nucleotide PCR product for the E6 cDNA, using one-twentieth of the total cDNA reaction mixture. To control for possible contamination by genomic DNA, parallel reactions were run using 0.175 μg of total RNA in the absence of the reverse transcriptase enzyme. Reaction mixtures were separated on a 4.5% NuSieve GTG-agarose gel (FMC BioProducts). Reaction mixtures were incubated with a 32P-labeled nucleotide consisting of the NF-κB consensus binding sequence (Santa Cruz Biotechnology) (0.5 μg) at 4°C for 20 min. Nucleoprotein complexes were subjected to electrophoresis on native 5% polyacrylamide gels, dried under vacuum, and exposed to BioMax MR film (Eastman Kodak Co.).

For the reporter gene assay, cells (4 x 10^5 well) were plated onto 24-well plates and allowed to adhere overnight. Cells were then co-transfected with pNF-κB-luciferase and pE-CMV-SEAP (for normalization of transfection efficiency) using Fugene 6 (Roche Molecular Biochemicals) according to the manufacturer’s protocol. 48 h post-transfection, the culture medium was changed, and TNF (2 ng/ml) was added to half the wells. Medium was again changed 16 h following TNF treatment. SEAP activity was measured in 20 μl of conditioned media using a commercially available assay kit (CLONTECH) following 24 h of SEAP secretion. The cells were then lysed, and the level of luciferase expression in cell lysates was determined using the Luciferase Assay System (Promega) according to the manufacturer’s protocol. Cells were washed twice with phosphate-buffered saline and lysed in 100 μl of lysis buffer (supplied with kit). Prepared Luciferase Assay Substrate (50 μl) was then mixed with 20 μl of cell lysate, and the luminescent signal was measured using a Turner luminometer. The level of luciferase expression was normalized to the level of SEAP activity and reported as a percentage of the activity in untreated cells.

RESULTS

Transfection of HPV 16 E6 into Mouse and Human Cells Provides Protection from TNF-triggered Cell Death—Our laboratory has previously shown that TNF-sensitive, mouse fibroblast LM cells are protected from TNF-induced cell death by the HPV 16 E6 protein in a p53-independent manner (32). In contrast to the LM cells, NIH3T3 mouse fibroblast cells must be treated with cycloheximide or actinomycin D to become sensitive to TNF. To determine whether E6 can protect NIH3T3 cells as well from TNF-induced apoptosis, NIH3T3 cells were co-transfected with pSV2neo and either the empty plasmid or the empty plasmid pSG5 or the E6 encoding plasmid pSG16E6. Pools of stably transfected cells were selected and either untreated or treated with TNF and/or cycloheximide. The extent of apoptosis was assessed using the Cell Death ELISA (Roche Molecular Biochemicals) (Fig. 1). The results demonstrate that cells transfected with pSG16E6 are protected from treatment with TNF and cycloheximide in comparison with those transfected with the empty plasmid.

To provide further evidence for the protective effects of E6 and to obtain sets of stably transfected, clonal human cell lines for further study, we transfected a plasmid encoding epitope-tagged E6 (HA-E6) into the human U937 cell line (histiocyte/monoocyte). An epitope-tagged, antisense version of E6 was used as a negative control. Following selection in the presence of G418, individual clones were isolated. Clones were screened...
for expression of the transfecting protein (HA-E6) by immunoblotting and tested for apoptosis in response to TNF. Although these cells were somewhat sensitive to TNF in the absence of cycloheximide, their sensitivity increases significantly in the presence of the protein synthesis inhibitor. Cycloheximide was therefore included in the culture medium in order to provide a more stringent test of the ability of E6 to protect cells from TNF. Untransfected U937 cells as well as a clone expressing the sense version of HA-E6 (U937E61) and a clone transfected with the antisense version of HA-E6 (U937AS) were therefore treated with either cycloheximide alone or cycloheximide plus TNF for 16 h, and the number of viable cells was measured by the MTT assay (Fig. 2A). As expected, the clone transfected with the antisense version of HA-E6 was as susceptible to TNF as was the parental line. However, the clone transfected with the sense version of HA-E6 experienced protection from TNF. Expression of the HA-E6 protein in the U937E61 cell line was confirmed by immunoprecipitation (Fig. 2B).

Similar experiments were performed with the U2OS cell line. As anticipated, expression of the sense version of HA-E6 (U2OSE62, U2OSE612, and U2OSE617), but not the antisense version of the protein (U2OSAS) provided protection from TNF-triggered cell death (Fig. 3A). Untransfected U2OS cells as well as the three clones transfected with HA-E6 were analyzed for expression of HA-E6 by immunoblotting and immunoprecipitation (Fig. 3B, top and middle panels). Whereas no reactive band was found in lysates of U2OS by either technique, as expected, clones U2OSE62 and U2OSE612 gave strong signals at the expected migration position for HA-E6. Clone U2OSE617 gave a weak signal by immunoblotting but no detectable signal in the immunoprecipitation analysis, suggesting that the amount of HA-E6 expressed in this clone was less than that expressed in clone U2OSE62 or U2OSE612. To confirm the expression of E6 in the U2OSE617 cells, reverse transcriptase-PCR was performed (Fig. 3B, bottom panel). Interestingly, even the more modest expression of E6 in U2OSE617 was sufficient to inhibit TNF-induced apoptosis.

Following DNA damage, the cellular level of the p53 tumor suppressor typically increases severalfold and activates genes involved in DNA repair, blockage of the cell cycle, and the induction of apoptosis, thus preventing the replication of damaged DNA. One of the best characterized activities of the E6 oncogene is to mediate the rapid degradation of the p53 tumor suppressor. To verify that the epitope-tagged HA-E6 protein is biologically active and able to degrade p53, we treated HA-E6-expressing and -nonexpressing cells with the DNA-damaging agent mitomycin C and measured the resulting p53 levels. When untransfected U2OS cells were treated with mitomycin C, the level of p53 increased ~10-fold (Fig. 3C). However, this increase in p53 was abolished in U2OS-derived cells expressing E6 (U2OSE62 and U2OSE612). Clone U2OSE617 expressed a reduced but still detectable level of p53 following mitomycin C treatment, most likely due to reduced levels of E6 in these cells (Fig. 3B). These results indicate that the transfected HA-E6, like wild-type E6, is able to significantly decrease the level of cellular p53.

**HPV 16 E6 Suppresses Caspase 3 Activation**—One possible explanation for the ability of E6 to protect expressing cells from TNF was that it interfered with the TNF-triggered apoptotic pathway. Therefore, we investigated whether E6 is capable of inhibiting caspase 3 activation. LM and LME6 cells were treated with TNF for various intervals and lysed. The lysates were assayed for caspase 3 activation using a colorimetric assay (Fig. 4A). The results demonstrate that TNF treatment results in activation of caspase 3 in LM cells, with a maximum level observed at about 12 h. However, no such activation was observed after TNF treatment of LME6 cells. Similar results were obtained in experiments using the U2OS and U2OSE612 cells (Fig. 4B). Caspase 3 was activated by TNF earlier in U2OS cells than in LM cells (3 versus 12 h), probably due to the inclusion of cycloheximide in the assays using U2OS and U2OSE612 cells. These results indicate that the influence of HPV 16 E6 on the TNF pathway occurs at or prior to the activation of caspase 3.
HPV 16 E6 Suppresses Caspase 8 Activation—Caspase 8 acts upstream of caspase 3 in the TNF-triggered apoptotic pathway. To determine whether E6 influences activation of caspase 8, we assayed its activity in U2OS and U2OSE612 cells before and after treatment with TNF plus cycloheximide (Fig. 4C). The results indicate that expression of E6 suppresses activation of caspase 8 in U2OS cells. Therefore, E6 blocks caspase 8 activation, suggesting that E6 blocks signals that emerge from the TNF receptor.

HPV 16 E6 Binds to the TNF R1—To examine the possibility that E6 binds directly to TNF R1, we immunoprecipitated proteins from lysates of U2OS cells stably expressing HAE6 using antibodies directed against either p53 or HA. Anti-p53 was chosen as an irrelevant antibody to be used as a negative control, since these cells do not express p53 due to E6 expression. Precipitated proteins were resolved by SDS-PAGE, transferred to a membrane, and immunoblotted using antibodies directed against either HA (closed bars) or HA-E6 (open bars). After incubation for 16 h, the percentage of viable cells was determined by the MTT assay. Measurements were made in triplicate, and the error bars represent the S.D. B, expression of HA-E6 in U2OS-derived clones. The indicated cells were lysed, and the expression of HA-E6 was detected by immunoblotting (top panel) and immunoprecipitation (middle panel), using anti-HA antibodies. Expression of HA-E6 in U2OSE617 was confirmed by reverse transcriptase-PCR, with lanes 1 and 3 serving as negative controls for possible contamination by genomic DNA (bottom panel). The arrows indicate the expected migration position of the indicated products. C, biological activity of HA-E6. The level of p53 was measured in the indicated clones before and after treatment with mitomycin C by ELISA. Measurements were made in duplicate, and the error bars represent the S.D.
pressing plasmid. Expression of CAT under these conditions indicates an interaction between the two test proteins. We also tested the binding of TRADD to TNF R1, since this well established binding interaction could serve as a positive control. In these experiments, neither plasmid alone was capable of inducing expression of CAT (data not shown). The results from the test plasmids show that there is a strong association between the death domain of TNF R1 and E6, comparable with that between the TNF R1 and TRADD (Fig. 5B, leftmost two bars).

The C-terminal 41 Amino Acids of TNF R1 Are Required for E6 Binding—The TNF R1 sequence contains two (E/D)LL(L/V)G motifs, shown by Elston et al. (37) to be an E6-binding motif. This sequence occurs twice, at amino acids 419–422 and 429–433 (PRREATLELLGRVRDMDLLGCL) of the death do-

FIG. 4. Caspase 3 and 8 activation is suppressed in E6-expressing cells. A, LM and LME6 cells were treated with TNF (1 ng/ml) for the times indicated and then lysed. Lysates were analyzed for caspase 3 activity using Ac-DEVD-pNA as substrate in the presence and absence of the caspase 3 inhibitor Ac-DEVD-CHO. The activity in wells containing the inhibitor was subtracted from that in wells lacking the inhibitor and then normalized for the amount of protein added to each well. Activity is expressed as the percentage of caspase activity in untreated LM cells. Each time point was measured in triplicate, and error bars represent the S.D. B, U2OS and U2OSE612 cells were treated with TNF (5 ng/ml) plus cycloheximide (5 μg/ml) for the times indicated and then lysed. Lysates were analyzed for caspase 3 activity as described for A. Activity is expressed as the percentage of caspase activity in untreated U2OS cells. Each point was measured in triplicate, and error bars represent the S.D. C, U2OS and U2OSE612 cells were treated with TNF (5 ng/ml) plus cycloheximide (5 μg/ml) for the times indicated and then lysed. Lysates were analyzed for caspase 8 activity using a colorimetric assay kit, with IETD-pNA as substrate and Ac-IETD-CHO as inhibitor. The activity in wells containing the inhibitor was subtracted from that in wells lacking the inhibitor and then normalized for the amount of protein added to each well. Activity is expressed as the percentage of caspase activity in untreated U2OS cells. Each point was measured in triplicate, and error bars represent the S.D.

FIG. 5. HPV 16 E6 binds to the TNF R1. A. TNF R1 co-immunoprecipitates with HA-E6. Lysates of U2OS cells stably expressing HA-E6 were immunoprecipitated with either anti-p53 DO-7 (lane 1) or anti-HA antibodies (lane 2). Following separation of the immunoprecipitated proteins by SDS-PAGE and transfer to membranes, the membranes were probed with either anti-TNF R1 (top) or anti-HA (bottom). The arrowhead shows the expected migration position of TNF R1. B, the C-terminal 41 amino acids of TNF R1 are required for binding to both E6 and to TRADD. Sequences encoding the death domain of TNF R1, the truncated version of the TNF R1 death domain, TRADD, and HPV 16 E6 were cloned into the bait or prey plasmids of a mammalian two-hybrid assay kit. The indicated combinations of plasmids were then transfected into U2OS cells, along with a reporter plasmid coding for CAT, and expression of the CAT gene was measured colorimetrically using a CAT-ELISA kit. The indicated values represent the mean of two independent experiments, with four measurements taken for each. Error bars represent the S.D. Inset, the truncated TNF R1 death domain is expressed in U2OS cells. Proteins from lysates of U2OS cells, either untransfected (lane 1) or transfected with a plasmid that encodes the truncated version of the TNF R1 death domain (pVPTNF R1 Δ DD) (lane 2), were separated by SDS-PAGE and transferred to a membrane, and the membrane was probed with antibodies directed against the activation domain. An arrow indicates the expected migration of the fused protein.
main. To test the possibility that the region containing this site might be required for TNF R1/E6 binding, DNA coding for the C-terminal 41 amino acids of TNF R1 was removed from the expression plasmid, and the binding of this truncated version of TNF R1 to both HPV 16 E6 and to TRADD was tested using the mammalian two-hybrid system (Fig. 5B, rightmost two bars). The results demonstrate that binding of the truncated version of TNF R1 to both HPV 16 E6 and TRADD is significantly reduced. Expression of the truncated version of the TNF R1 death domain in these cells was verified by immunoblotting (inset). Thus, these results suggest that the binding site for both HPV 16 E6 and for TRADD is at the C-terminal, cytoplasmic tail of TNF R1.

HA-E6 Interferes with Cell Death Triggered by Transfection of TNF R1—Transfection of cells with TNF R1 leads to cell death in a number of cell types. If HPV 16 E6 blocks signal transduction by binding to TNF R1, E6 should interfere with apoptosis triggered by TNF R1 transfection as well as that induced by TNF. To test this prediction, parental U2OS cells and the E6-expressing clone U2OSE612 were transiently transfected with either the full-length (TNF R1 DD) or truncated (TNF R1 Δ DD) versions of the TNF R1 death domain, and cell viability was measured by the MTT assay (Fig. 6). As anticipated, the truncated version of TNF R1 was incapable of inducing significant cell death in either cell line, consistent with the proposed role of the C-terminal domain in binding to TRADD. The full-length version of the TNF R1 death domain did induce significant cell death in the parental U2OS cells. However, the U2OSE612 clones experienced complete protection from cell death induced by overexpression of TNF R1 death domain, consistent with the proposed role of E6 in blocking TNF R1-mediated signal transduction.

HPV 16 E6 Does Not Decrease the Level of TNF R1—Degradation of some, but not all, of the proteins to which E6 binds is accelerated in the presence of E6, resulting in a significantly decreased steady-state level of the protein. For example, the binding of HPV 16 E6 to E6AP and p53 promotes the rapid degradation and loss of p53 (38), while the binding of E6 to IRF-3 does not result in either the ubiquitination or degradation of IRF-3 (14). To examine whether E6 decreases the level of cellular TNF R1, we compared the steady-state levels of TNF R1 by immunoblotting in cells expressing or not expressing E6 (data not shown) and found that the levels of TNF R1 are not significantly lower in cells expressing E6.

HPV 16 E6 Inhibits TNF R1/TRADD Interactions—To determine the mechanism by which the binding of HPV 16 E6 to TNF R1 inhibits TNF-induced apoptosis, we examined whether E6 alters the binding of TNF R1 to TRADD, one of the earliest steps in TNF-mediated apoptotic signaling. This seemed likely, since the C-terminal 41 amino acids of TNF R1 are required for both E6 and TRADD binding. If E6 blocks the binding of TNF R1 to TRADD, the TNF R1/TRADD binding signal should be reduced in the presence of E6. To test this prediction, we used the mammalian two-hybrid system to examine TNF R1/TRADD binding in three cell lines, the parental U2OS cells (E6 negative) and the stably-transfected, E6-expressing U2OS derivatives U2OSE62 and U2OSE66 (Fig. 7). In these experiments, the binding of TNF R1 to TRADD was significantly reduced in the presence of E6. In a control experiment, interactions between p53 and the SV40 T antigen were not reduced in U2OSE66 cells in comparison with U2OS cells (data not shown), indicating that the decrease in TNF R1/TRADD binding in these cells cannot be accounted for by alterations in the capacity of these cells to support the two-hybrid system. Therefore, these data are consistent with a model in which binding of E6 to TNF R1 interferes with the recruitment of TRADD and subsequent assembly of the DISC and generation of proapoptotic signals.

HPV 16 E6 Is Not a Major Inhibitor of the Mitochondrial Apoptotic Pathway—Our finding that E6 binds to TNF R1 and inhibits signaling through TRADD does not exclude the possibility that it may affect other apoptotic pathways as well. Since TNF has been shown to activate the mitochondria-mediated apoptotic pathway of at least some cells (39, 40), it seemed possible that E6 might block TNF-induced apoptosis by inhibiting this pathway. To address this possibility, we treated E6-expressing and control cells for 24 h with ceramide (25 μM), which is an activator of the mitochondria-mediated apoptotic pathway.
pathway. Cell survival was monitored by the MTT assay (Fig. 8). Ceramide induced equivalent levels of apoptosis in E6-expressing and control cell lines, providing evidence that E6 does not inhibit mitochondria-mediated apoptosis.

**HPV 16 E6 Does Not Up-regulate the NF-κB-mediated Protective Pathway**—When TNF binds to its receptor, both pro-apoptotic and prosurvival pathways are activated. The pro-apoptotic pathway proceeds via the adaptor proteins TRADD and FADD, through activation of initiator caspases such as caspase 8, activation of effector caspases such as caspase 3, cleavage of cellular substrates, and finally, apoptosis. The protective pathway also begins with TRADD but then diverges and requires additional proteins, such as RIP, TRAF2, and NF-κB (reviewed in Ref. 41). It was therefore possible that E6 might exert its antiapoptotic effects by up-regulating the protective, NF-κB pathway. To test this possibility, we treated LM and LME6 cells with TNF (1 ng/ml) and assessed NF-κB activation by electrophoretic mobility shift assays as previously described (35, 36) (Fig. 9A). The results demonstrate that NF-κB activation does occur in LM cells, with two waves of activation occurring at about 20 min and then again at about 4 h. These results suggest that whereas NF-κB activation does occur in LM cells, it is inadequate to prevent TNF-induced apoptosis. In addition, the level of NF-κB activation does not differ significantly between the two cell lines, providing evidence that up-regulation of NF-κB plays at most a minimal role in the mechanism by which E6 protects cells from apoptosis induced by TNF.

To confirm these results, we used a reporter gene assay to measure the capacity of NF-κB to enhance transcription of luciferase from a reporter plasmid before and after TNF treatment of LM and LME6 cells. Consistent with our previous results, we found that TNF treatment induced NF-κB-directed luciferase expression in both cell lines (Fig. 9B). Therefore, the capacity of E6 to protect cells from TNF-induced apoptosis cannot be explained by a significant up-regulation of the NF-κB-mediated protective pathway.
DISCUSSION

Our previous work had shown that E6 can protect expressing cells from TNF, and the purpose of this study was to identify the molecular mechanism(s) underlying this protection. We found that HPV 16 E6 binds to TNF R1 and affects the transmission of proapoptotic signals triggered by TNF. The binding of E6 to TNF R1 was demonstrated by both co-immunoprecipitation and the mammalian two-hybrid system. The binding is significant, with the signal from the two-hybrid system approximating that observed for the well established TNF R1/TRADD interaction. We hypothesize that the binding of E6 to TNF R1 hinders the sequential interactions required to form the DISC. This inhibition in turn would be predicted to inhibit activation of initiator caspases (such as caspase 8), leading to suppression of effector caspases (such as caspase 3) and blockade of apoptosis. Our results are consistent with this model. The experiment shown in Fig. 7 indicates that TNF R1/TRADD binding is reduced in the presence of E6, and the experiments shown in Figs. 1–4 and 6 provide evidence that E6 blocks transmission of apoptotic signals. Hence, E6 prevents both TNF R1/TRADD binding and proapoptotic signal transduction.

This work demonstrates that the C-terminal 41 amino acids of TNF R1 are required for E6 binding (Fig. 5), TRADD binding (Fig. 5), and generation of proapoptotic signals (Fig. 6). This suggests that the binding sites for TRADD and HPV 16 E6 are topologically proximate. The region of TNF R1 critical for generation of apoptotic signaling was previously localized to an 80-amino acid region near the C terminus of the protein (42). Our results are consistent with these previous findings and add the functions of TRADD and E6 binding to this general region.

The capacity of E6 to bind to the TNF R1 death domain may be due to the presence of the previously identified E6-binding motif, IE/DIL/L/VG, since two copies of this motif are localized in the C-terminal 41 amino acids. The existence of these sequences in a region of TNF R1 required for E6 binding provides further evidence for the E6 binding affinity of this motif. It should be noted, however, that this motif is not present in all E6-binding proteins. Therefore, there must be additional binding sites with affinity for E6.

We were able to demonstrate that protection against TNF-induced apoptosis by HPV 16 E6 occurs in cells of different species (mouse and human) and tissues (fibroblast, osteosarcoma, and histiocyte/monocyte) (Figs. 1–3) (32), providing evidence of its generality. These results differ from those obtained earlier, where it was reported that either the bovine (43) or the human (44, 45) version of E6 sensitized cells to TNF-triggered cell death. This difference probably resulted from alternate signaling pathways being engaged in the two systems, with our studies finding clear evidence of caspase activation following TNF treatment, indicating an apoptotic form of cell death, whereas Liu et al. (44) determined the mode of death to be necrosis. Interestingly, the E6 in our system did cause p53 degradation, as expected, whereas p53 was not degraded in the previous system. Binding of E6 to TNF R1 could conceivably result in either sensitization or resistance to TNF, depending on how the presence of E6 affects downstream events. Under some conditions, blocking the apoptotic pathway, as we have shown E6 to do, may shift cells into a necrotic pathway (46). Relevant factors influencing this shift could include the cell type, the dose of TNF, and the amount of E6 expressed. Following inhibition of the apoptotic pathway by E6, differences in such factors between the experimental systems could result in either cellular resistance, as we have found, or in a shift to necrosis, such as that observed by Liu et al. (44).

Interestingly, the results of our experiments using clone U2OSE617 suggest that the level of E6 expression is important in determining its biological activities. The experiment shown in Fig. 3B indicates that this clone expresses a low, although detectable, level of HA-E6. This level of E6 expression is sufficient to completely protect these cells from TNF-induced apoptosis (Fig. 3A) yet insufficient to completely eliminate the DNA damage-induced increase in cellular p53 (Fig. 3C).

We were unable to detect significant differences between E6-expressing and E6-negative cells in their response to ceramide, which excluded significant interference by E6 in the pathway leading from mitochondrial activation to apoptosis. Furthermore, we were unable to detect significant differences in the induction of the NF-κB-mediated protective pathway in E6-expressing and E6-negative cells. These results emphasize the importance of E6/TNF R1 binding in the observed modulation of TNF sensitivity in E6-expressing cells.

Interestingly, although E6 binds to the TNF R1 and inhibits transmission of the apoptotic signal, it does not abolish activation of NF-κB (Fig. 9). In this respect, E6 may act in a manner similar to that observed for the hepatitis C virus core protein (47). Also, the sensitization effect of E6 reported by Liu et al. did not occur by altering TNF-induced NF-κB activation (44). One possible explanation for our finding is that NF-κB is activated by TNF using a mechanism that does not require TNF R1/TRADD interactions.

To survive and propagate, viruses have developed numerous ways to avoid destruction by the host immune system. The means by which they have done so are now known to encompass a wide ranging and diverse set of molecular mechanisms that can target several different steps of multiple immune pathways. Some viruses encode proteins that disable only one or a few of these mechanisms. Others encode one or several proteins that can systematically target cellular defenses at several levels (reviewed in Ref. 48).

Several viruses have developed strategies to block TNF-mediated host responses. Adenovirus encodes four proteins that prevent TNF-mediated apoptosis (49–53), and three adenovirus proteins cooperate to prevent apoptosis induced by the closely related molecule, TRAIL (54). Some poxviruses encode secreted proteins that are similar to the TNF receptor (55–57), and the binding of the Shope fibroma T2 protein to TNF inhibits the cytokine from binding to its receptor (58). The poliovirus protein 3A inhibits TNF-induced apoptosis by eliminating cell surface expression of the TNF receptor (59), and the hepatitis C virus core protein binds to the death domain of TNF R1 and suppresses signaling from TRADD (47). Our results provide evidence that HPV 16 also blocks TNF-mediated apoptosis and that it does so by blocking interactions between TNF R1 and TRADD.

Including TNF R1, the number of E6-binding cellular proteins has now reached over a dozen. With few exceptions, most notably the E6/E6AP interaction, the roles of these binding interactions in virus replication and virus-mediated pathology are incompletely understood. Papillomaviruses are persistent viruses that remain in their hosts for long periods of time. The papillomavirus-specific immune response is either weak or undetectable, and little or no inflammatory response is elicited by papillomavirus infection. The capacity of E6 to protect cells from TNF may be an important factor in this lack of a host inflammatory response and thus contribute both to the persistence of this virus and to its oncogenic potential. Moreover, our finding that TNF R1 is a binding partner for E6 suggests additional approaches for the development of therapeutic agents for cervical cancer. It is possible that small molecules capable of interfering with E6/TNF R1 binding might increase the sensitivity of E6-expressing cervical cancer cells to either
endogenous or therapeutic doses of TNF or to conventional chemotherapeutic agents that lead to TNF release.

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The Human Papillomavirus 16 E6 Protein Binds to Tumor Necrosis Factor (TNF) R1 and Protects Cells from TNF-induced Apoptosis

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